UNIVERSIDADE FEDERAL DE LAVRAS

PRISCILA EMANUELA DE SOUZA

LIVING IN THE SUBTERRANEAN TIME: EXPLORING BIOLOGICAL RHYTHMS IN CAVE INVERTEBRATES

LAVRAS – MG
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Dissertação apresentada à Universidade Federal de Lavras, como parte das exigências do Programa de Pós-Graduação em Ecologia Aplicada, área de concentração em Ecologia e Conservação de Recursos Naturais em Paisagens Fragmentadas e Agroecossistemas, para a obtenção do título de Mestre.

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VIVENDO NO TEMPO DO SUBTERRÂNEO: EXPLORANDO RITMOS BIOLÓGICOS EM INVERTEBRADOS DE CAVERNAS

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RESUMO GERAL

Os manuscritos apresentados nesta dissertação emergiram da necessidade de ampliar o conhecimento das lacunas existentes na área da cronobiologia em cavernas, especialmente com invertebrados, os quais exibem uma diversidade de morfologias, fisiologias e comportamentos associados aos diferentes níveis de adaptação a esses ambientes cíclicos. Nesse contexto, esses ecossistemas são caracterizados pela baixa variabilidade abiótica e pela ausência de ciclos claro-escuro. Dessa forma, os organismos manifestam seus ritmos biológicos de maneiras diversas individualmente e entre os diferentes táxons, sem apresentar um padrão claro entre as categorias ecológico-evolutivas que compõem a fauna cavernícola. Essa percepção serviu como base para as hipóteses investigadas no segundo e terceiro manuscritos. O objetivo geral desta dissertação foi não apenas entender a manifestação dos ritmos biológicos na fauna de invertebrados cavernícolas, mas também entender como esses ritmos podem variar entre diferentes classes taxonômicas. A literatura destaca a adaptação do relógio biológico em cavidades naturais subterrâneas, onde fatores como luz, temperatura e alimentação desempenham papel significativo na regulação dos ritmos biológicos. Estudos demonstram a evolução de relógios não circadianos devido à ausência de ciclos diurnos, resultando em ciclos infradianos ou ultradianos. Apesar dos avanços realizados, há ainda lacunas a serem exploradas na pesquisa sobre a ritmicidade de invertebrados em ambientes subterrâneos. O primeiro manuscrito, um artigo de revisão, se concentrou em quatro questões principais: (i) as regiões geográficas mais estudadas em relação à cronobiologia de invertebrados de cavernas, (ii) os táxons mais frequentemente estudados, (iii) as características evolutivas dos táxons documentados e (iv) a manifestação do comportamento rítmico dos invertebrados em ambientes sem ciclos e sua correlação com a morfologia funcional. O segundo manuscrito surgiu de uma lacuna na pesquisa sobre o comportamento rítmico de isópodes em ecossistemas subterrâneos, enfatizando a importância de investigar esse grupo pouco explorado em relação aos padrões de atividade locomotora. Nesse contexto, a pesquisa concentrou-se na possibilidade de perda de elementos rítmicos devido à evolução convergente em ambientes subterrâneos. Foi formulada a hipótese de que diferentes espécies de isópodes semiaquáticos obrigatórios apresentam padrões aperiódicos em sua atividade locomotora, devido à falta de ciclos de luz nas cavernas e à variabilidade térmica, fatores cruciais para a regulação dos ritmos biológicos. Os resultados indicaram sincronização do ritmo circadiano dentro do tratamento LD, embora sem padrões claramente definidos sob condições constantes de luz e escuridão. O terceiro manuscrito surgiu da lacuna nos estudos cronobiológicos envolvendo amblipígios e investigou possíveis diferenças nos padrões rítmicos entre espécies troglófilas (capazes de estabelecer populações tanto dentro quanto fora de cavernas) e troglóbias (obrigatoriamente confinadas a cavernas). Foi identificada variabilidade no comportamento rítmico, mas sem diferenças significativas entre os grupos ou espécies; as distinções foram individuais. Esses achados sublinham a necessidade de ampliar a pesquisa sobre ritmicidade em cavernas, abrangendo mais áreas geográficas e grupos taxonômicos. É crucial investigar os ritmos biológicos para entender as adaptações subterrâneas, especialmente frente a ameaças como mudanças climáticas e perda de habitat, contribuindo para a conservação e avançando no conhecimento ecológico e evolutivo desses ambientes.

Palavras-chave: Cronobiologia; ritmo circadiano; aperiodicidade; caverna; invertebrados.

ABSTRACT

The manuscripts presented in this dissertation emerged from the need to expand knowledge regarding existing gaps in the field of chronobiology in caves, especially concerning invertebrates, which exhibit a diversity of morphologies, physiologies, and behaviors associated with different levels of adaptation to these cyclic environments. In this context, these ecosystems are characterized by low abiotic variability and the absence of clear-dark cycles. Consequently, organisms manifest their biological rhythms in diverse ways individually and among different taxa, without presenting a clear pattern among the ecological-evolutionary categories that compose cave fauna. This perception served as the basis for the hypotheses investigated in the second and third manuscripts. The general objective of this dissertation was not only to understand the manifestation of biological rhythms in cave invertebrate fauna but also to understand how these rhythms may vary among different taxonomic classes. The literature highlights the adaptation of the biological clock in underground natural cavities, where factors such as light, temperature, and food play a significant role in regulating biological rhythms. Studies demonstrate the evolution of non-circadian clocks due to the absence of diurnal cycles, resulting in infradian or ultradian cycles. Despite the progress made, there are still gaps to be explored in research on the rhythmicity of invertebrates in underground environments. The first manuscript, a review article, focused on four main questions: (i) the most studied geographic regions regarding the chronobiology of cave invertebrates, (ii) the most frequently studied taxa, (iii) the evolutionary characteristics of documented taxa, and (iv) the manifestation of rhythmic behavior of invertebrates in cycle-less environments and its correlation with functional morphology. The second manuscript emerged from a gap in research on the rhythmic behavior of isopods in underground ecosystems, emphasizing the importance of investigating this little-explored group regarding patterns of locomotor activity. In this context, the research focused on the possibility of losing rhythmic elements due to convergent evolution in underground environments. The hypothesis was formulated that different species of obligatory semiaquatic isopods present aperiodic patterns in their locomotor activity due to the lack of light cycles in caves and thermal variability, crucial factors for the regulation of biological rhythms. The results indicated synchronization of the circadian rhythm within the LD treatment, albeit without clearly defined patterns under constant light and darkness conditions. The third manuscript arose from the gap in chronobiological studies involving amblypygids and investigated possible differences in rhythmic patterns between troglophile species (capable of establishing populations both inside and outside caves) and troglobite species (obligatorily confined to caves). Variability in rhythmic behavior was identified, but there were no significant differences between groups or species; the distinctions were individual. These findings underline the need to expand research on rhythmicity in caves, encompassing more geographical areas and taxonomic groups. It is crucial to investigate biological rhythms to understand subterranean adaptations, especially in the face of threats like climate change and habitat loss, contributing to conservation and advancing the ecological and evolutionary understanding of these environments.

Keywords: Chronobiology; circadian rhythm; aperiodicity; cave; invertebrates.

INDICADORES DE IMPACTO

A dissertação, situada no âmbito do meio ambiente, foca na investigação dos ritmos biológicos em animais invertebrados de caverna. Partindo da hipótese de que o isolamento em ambientes de escuridão permanente pode provocar alterações nos mecanismos de regulação dos osciladores circadianos, como o sistema visual, a temperatura corporal e a produção de melatonina, o estudo busca identificar as interações entre ritmos biológicos, ambiente e características morfológicas das espécies cavernícolas. Para embasar essa pesquisa, foi conduzida uma revisão bibliográfica rigorosa, utilizando as plataformas Web of Science e Google Scholar. Esta etapa resultou na identificação de mais de 480 estudos, conforme detalhado no Artigo I, com foco em palavras-chave pertinentes ao tema. Dentre esses trabalhos, somente 44 foram selecionados, após criteriosa avaliação que considerou os objetivos específicos da pesquisa. A maioria dos estudos excluídos não atendia às diretrizes da pesquisa, sendo majoritariamente estudos com vertebrados, como peixes, morcegos, anfíbios e humanos, ou investigações que não se relacionavam diretamente com ritmos biológicos, como descrições de espécies, aspectos culturais, geoarqueologia, geocronologia e paleoclima, entre outros. Para enriquecer o estudo, contamos com a colaboração de Bruno da Silva Brandão Gonçalves, especialista em processamento de sinais biológicos e ritmicidade circadiana. Sua expertise foi fundamental para a análise detalhada dos dados e para um entendimento mais aprofundado da área de estudos, facilitando a identificação de lacunas.

O estudo evidenciou a necessidade urgente de preencher as lacunas existentes na pesquisa sobre ritmos biológicos em invertebrados de caverna, abrangendo diversas regiões geográficas e uma ampla gama de grupos taxonômicos. É alarmante a carência de estudos em regiões como o continente africano, que abriga quase 300 espécies estritamente cavernícolas e de habitat aquático, sem investigações na área de cronobiologia. Além disso, é vital incorporar pesquisas que explorem os aspectos temporais, evolutivos e ecológicos relacionados ao desacoplamento ou sincronização com os ritmos biológicos. Compreender as adaptações da vida subterrânea e elucidar os mecanismos de regulação dos ritmos biológicos em ambientes subterrâneos torna-se essencial, especialmente diante das ameaças globais emergentes, como mudanças climáticas e perda de habitat. Este conhecimento é crucial para a preservação dos ecossistemas subterrâneos e para assegurar sua resiliência frente a essas ameaças. Vale ressaltar que os impactos alcançados por este trabalho estão em consonância com os 17 Objetivos de Desenvolvimento Sustentável (ODS) da Organização das Nações Unidas (ONU).

IMPACT INDICATORS

The dissertation, situated in the context of the environment, focuses on the investigation of biological rhythms in cave invertebrates. Based on the hypothesis that isolation in permanent darkness environments can cause alterations in the mechanisms regulating circadian oscillators, such as the visual system, body temperature, and melatonin production, the study seeks to identify the interactions between biological rhythms, environment, and morphological characteristics of cave species. To support this research, a rigorous bibliographic review was conducted using the Web of Science and Google Scholar platforms. This stage resulted in the identification of more than 480 studies, as detailed in Article I, focusing on keywords relevant to the topic. Among these works, only 44 were selected after careful evaluation considering the specific objectives of the research. Most of the excluded studies did not meet the research guidelines, mainly being studies with vertebrates, such as fish, bats, amphibians, and humans, or investigations that were not directly related to biological rhythms, such as species descriptions, cultural aspects, geoarchaeology, geochronology, and paleoclimate, among others. To enrich the study, we collaborated with Bruno da Silva Brandão Gonçalves, an expert in biological signal processing and circadian rhythmicity. His expertise was essential for the detailed analysis of the data and for a deeper understanding of the study area, facilitating the identification of gaps.

The study highlighted the urgent need to fill existing gaps in research on biological rhythms in cave invertebrates, covering various geographic regions and a wide range of taxonomic groups. It is alarming the lack of studies in regions such as the African continent, which houses nearly 300 strictly cave-dwelling species with aquatic habitats, without investigations in the field of chronobiology. Moreover, it is vital to incorporate research that explores the temporal, evolutionary, and ecological aspects related to the decoupling or synchronization with biological rhythms. Understanding the adaptations of underground life and elucidating the mechanisms regulating biological rhythms in underground environments becomes essential, especially in the face of emerging global threats, such as climate change and habitat loss. This knowledge is crucial for the preservation of underground ecosystems and to ensure their resilience against these threats. It is worth noting that the impacts achieved by this work are in line with the 17 Sustainable Development Goals (SDGs) of the United Nations (UN).

SUMÁRIO

PRIMEIRA PARTE

INTRODUÇÃO GERAL

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Esta dissertação investiga o conhecimento dos ritmos biológicos em animais invertebrados de caverna, especialmente aqueles mais intimamente ligados à adaptação aos ambientes cavernícolas, tais como os troglóbios (espécies obrigatoriamente adaptadas às cavernas). Partindo da hipótese de que o isolamento em ambientes de permanente escuridão induz alterações nos mecanismos de regulação dos osciladores circadianos, como o sistema visual, a temperatura corporal e a produção de melatonina, o trabalho tem como objetivo identificar as relações entre ritmos biológicos, ambiente e morfologia (troglomorfismos) das espécies cavernícolas.

Para contextualizar o estado atual da pesquisa sobre ritmicidade em cavernas e compreender seu comportamento na fauna subterrânea brasileira, foram elaborados três manuscritos. O primeiro consistiu em uma revisão do conhecimento mundial sobre os ritmos biológicos em invertebrados subterrâneos, destacando os grupos mais estudados e a incidência dos estudos. O segundo manuscrito investigou o ritmo locomotoa atividade locomotora de isópodes anfíbios da família Styloniscidae, todos eles troglóbios, presentes em cavernas dos estados de Minas Gerais e Bahia. O terceiro manuscrito avaliou se o grau de especialização dos animais em relação ao meio em que vivem e a presença de elementos rítmicos exercem influência sobre o padrão de atividade locomotora de espécies troglóbias e troglófilas de *Charinus*.

O primeiro manuscrito ou artigo I, submetido e escrito conforme as normas do periódico “Chronobiology International”, revisou a cronobiologia de invertebrados em ecossistemas subterrâneos, enfatizando a importância dos relógios circadianos na coordenação de eventos biológicos. Foram analisados 44 estudos selecionados de mais de 480, revelando uma diversidade taxonômica significativa, com foco principal em espécies terrestres como coleópteros. Observou-se que espécies troglóbicas tendem a apresentar comportamento aperiódico, enquanto os troglófilos estão associados a uma expressão rítmica robusta. A atividade locomotora foi o aspecto mais estudado, mas algumas lacunas de informação foram identificadas, principalmente em estudos sob condições de luz constante. A revisão destaca a necessidade de expandir a pesquisa cronobiológica globalmente para uma melhor compreensão dos ritmos biológicos subterrâneos, essenciais para a preservação da resiliência dos ecossistemas subterrâneos diante de ameaças como as alterações climáticas e a perda de habitat.

O segundo manuscrito ou artigo II, submetido e escrito conforme as normas do periódico “Journal of Biological Rhythms”, abordou a possível conservação do ritmo circadiano na atividade locomotora em isópodes troglóbios da família Styloniscidae, destacando a influência da presença de luz nesta atividade. As espécies *Iuiuniscus iuiuensis*, *Xangoniscus aganju*, *Xangoniscu*s *santinhoi*, *Xangoniscu*s sp.1 e *Xangoniscu*s sp.2 foram estudadas em diferentes condições de luz: escuridão constante (DD), luz constante (LL) e ciclos claro-escuro (LD). Descobriu-se que todas as espécies apresentaram predominantemente ritmo infradiano e circadiano fraco para DD e LL, enquanto sob LD, houve sincronização pelos regimes de luminosidade. As variações comportamentais foram observadas entre as espécies, com algumas apresentando atividade diurna e outras noturna. O estudo ressalta a importância de compreender como os organismos se adaptam a ambientes cíclicos e não cíclicos e a necessidade de explorar os mecanismos subjacentes envolvidos na atividade circadiana.

Já o terceiro manuscrito ou artigo III, submetido e escrito conforme as normas do periódico “Journal of Zoology”, avaliou o padrão de atividade locomotora de espécies troglóbias e troglófilas de *Charinus*, considerando seu grau de especialização e a presença de elementos rítmicos. Utilizou-se a mesma metodologia citada acima referente ao artigo II. Não houveram distinções claras em seus ritmos entre espécies troglófilas e troglóbias. Além disso, observou-se uma complexa dinâmica envolvida na determinação e diversificação dos ritmos circadianos dentro destas espécies.

Essa dissertação engloba uma área em que são encontrados pouquíssimos estudos que trabalharam com dados comportamentais de invertebrados cavernícolas que envolvem padrões de atividade (Friedrich, 2013; Beale *et al*., 2016).

As cavernas constituem ecossistemas que oferecem uma diversidade de microambientes, desde zonas de entrada com escuridão parcial até locais de escuridão total (afótica), caracterizados por variações ambientais praticamente ausentes ao longo do dia e mínimas ao longo do ano (Poulson and White, 1969). Em comparação com os ambientes de superfície, as cavernas proporcionam condições mais estáveis (Culver and Pipan, 2009) e, portanto, são consideradas arrítmicas (Beale *et al*., 2016). Esses ambientes abrigam uma fauna característica, adaptada à vida subterrânea, apresentando morfologias, fisiologias, modos de reprodução, ritmos de atividade e comportamentos distintos daqueles observados em animais que habitam a superfície, sujeitos a ciclos diários de claro e escuro (Soriano-Morales *et al*., 2013).

Os ambientes subterrâneos são geralmente considerados os menos propensos a apresentar ritmos cíclicos entre os habitats com essa característica (Abhilash *et al.* 2017).

Contudo, a persistência de ritmos em organismos que habitam cavernas nos leva a questionar sua finalidade e relevância, mesmo na ausência de uma coordenação direta da fisiologia do animal com o ambiente externo (Beale *et al.*, 2016). Os ritmos circadianos em ambientes desprovidos de ritmo são vestigiais, com diferentes espécies mantendo diferentes níveis de funcionalidade (Pasquali and Sbordoni, 2014; Beale *et al.*, 2016).

Os relógios circadianos desempenham um papel crucial na coordenação temporal de processos internos e na separação temporal de atividades incompatíveis, de acordo com a hipótese da vantagem intrínseca (Nikhil and Sharma, 2017). Essa ideia é sustentada pela observação de múltiplos ritmos diários em escalas biológicas e na coordenação temporal.

Em geral, o comportamento rítmico dos invertebrados reflete sua habilidade de adaptação e sincronização de atividades com pistas ambientais, destacando a complexa relação entre os relógios biológicos internos e os fatores ambientais externos (Brady, 1981).

Considerando a relevância desses ambientes e as lacunas de conhecimento que os cercam, estudos sobre ritmicidade em animais de cavernas podem desempenhar um papel fundamental na compreensão dos mecanismos intrínsecos (processos biológicos internos que regulam os ritmos biológicos (diários e circadianos, por exemplo) e extrínsecos (fatores externos que influenciam os ritmos circadianos, conhecidos como zeitgeber), assim como no valor adaptativo dessas características. Em cavernas, onde os fatores externos ou abióticos exercem pouca influência sobre os relógios biológicos dos animais que nelas habitam, devido à sua alta estabilidade, ainda permanece desconhecido um padrão que permita compreender como esses processos ocorrem e se manifestam em diferentes grupos taxonômicos.

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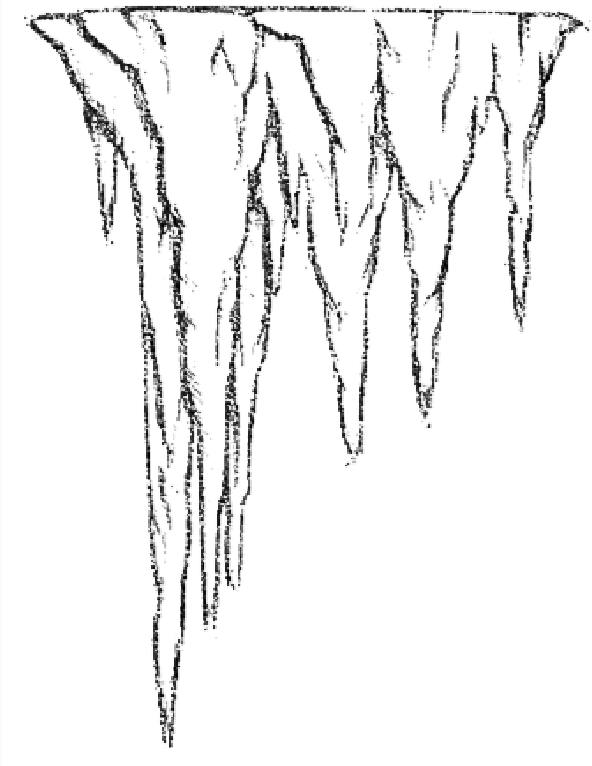
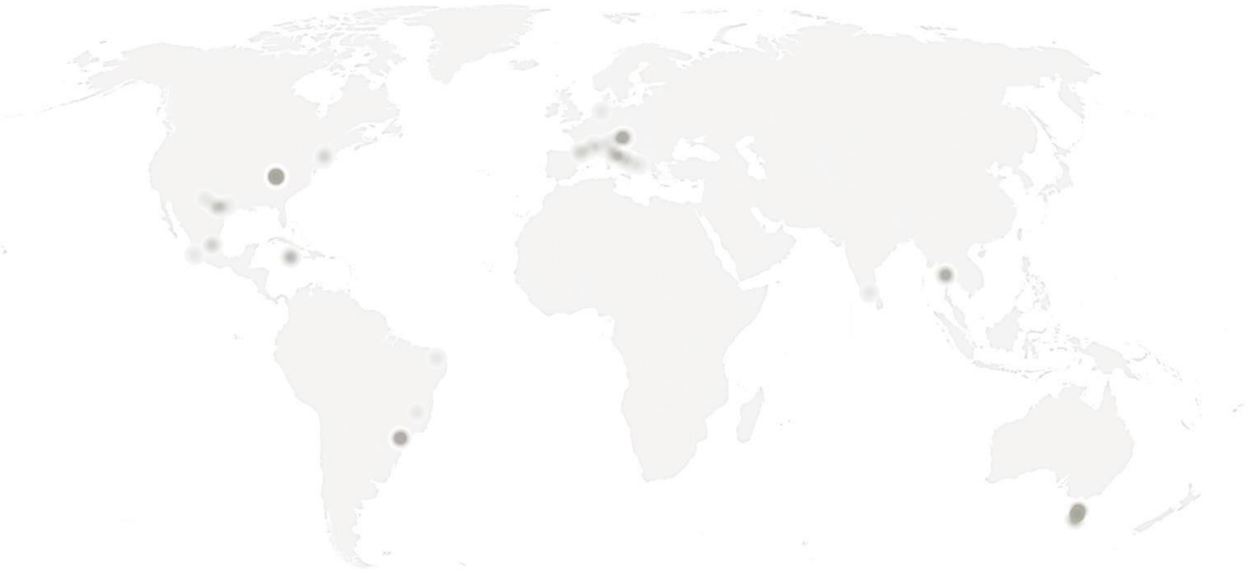


SEGUNDA PARTE

ARTIGOS



ARTIGO I

THE TICKING CLOCK IN THE DARKNESS: REVIEW OF BIOLOGICAL RHYTHMS IN CAVE INVERTEBRATES

ARTIGO I

Este manuscrito foi elaborado em sua versão preliminar, seguindo as diretrizes estabelecidas pela revista C*hronobiology International*

The ticking clock in the darkness: review of biological rhythms in cave invertebrates

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Abstract

Circadian clocks, internal mechanisms generate the circadian rhythms, play a crucial role in coordinating biological events with day-night cycles. In light-deprived environments such as caves, species, particularly isolated obligatory troglobites, may exhibit evolutionary adaptations in biological rhythms due to light exposure. To explore rhythm expression in these settings, we conducted a comprehensive literature review on invertebrate chronobiology in global subterranean ecosystems, analyzing 44 selected studies out of over 480 identified as of September 2023. These studies revealed significant taxonomic diversity, primarily among terrestrial species like Coleoptera, with research concentrated in the United States, Italy, France, Australia, and Brazil, and a notable gap in African records. Troglobite species displayed a higher incidence of aperiodic behavior, while troglophiles showed a robust association with rhythm expression. Locomotor activity was the most studied aspect (>60%). However, approximately 4% of studies lacked information on periodicity or rhythm asynchrony, and limited research under constant light conditions hindered definitive conclusions. This review underscores the need to expand chronobiological research globally, encompassing diverse geographical regions and taxa, to deepen our understanding of subterranean biological rhythms. Such insights are crucial for preserving the resilience of subsurface ecosystems facing threats like climate change and habitat loss.

Keywords: cave; biological clock; rhythms; invertebrates; cave-dwelling; arrhythmia

Introduction

Caves are unique ecosystems, characterized by their asynchrony when compared to external habitats and the presence of diverse microhabitats that vary in terms of darkness and climatic stability (Poulson and White 1969; Oda et al. 2000). These environments harbor a specialized subterranean fauna, notably distinct from surface-dwelling species (Soriano-Morales et al. 2013). Furthermore, caves partially isolated from the surface, present unique opportunities for ecological and evolutionary studies, as well as for understanding the adaptation of biological rhythms (Poulson and White 1969; Mammola 2019). Such opportunities arise from their unique traits as the absence of constant light or light-dark cycles, high humidity, low availability of trophic resources, and a consistent temperature close to the surface's annual average (Howarth 1983; Beale et al. 2016).

Animals that inhabit caves can be classified into four ecological-evolutionary categories, following the initial proposal by Schinner-Racovitza (1907) and the subsequent review by Barr (1967). Accidental organisms are those devoid of any pre-adaptations to survive in these systems, consequently being highly susceptible to becoming trapped when entering caves, resulting in death consequently. Trogloxenes are temporary residents, seeking shelter in caves during specific periods (Culver and Pipan 2009). Such individuals display a regular behavior of moving to the surface in search of food. This activity is associated with circadian patterns that follow the light-dark (LD) cycle, meaning they can synchronize their movements and activities with the daily cycle of light and darkness, influencing their patterns of movement between the subterranean and the surface (Gnaspini et al. 2003). Troglophiles can complete their life cycles in both epigean (surface) and hypogean (cave) environments and may exhibit variations in the amplitude of their circadian rhythm, potentially lacking clearly defined rhythmic patterns (Pasquali and Sbordoni 2014; Menna-Barreto and Trajano

2015). Finally, troglobitic species are restricted to caves and are obligate inhabitants, completing their entire life cycles exclusively within these cave habitats (Culver and Pipan 2009; Soriano-Morales et al. 2013; Moldovan et al. 2018). In this category, animals also exhibit variability in rhythmic responses, with arrhythmic behavior being a common occurrence as well (Trajano et al. 2009).

The light-dark cycle (LD) is the external stimulus that most significantly affects many species (Aschoff 1981; Oda et al. 2000), in addition to temperature and humidity (Trajano et al. 2009), and feeding stimulus (Gnaspini et al. 2003). In isolated populations, as those found in caves, circadian rhythms (from latin words *circa* = approximately, *dien*= day), as well as traits that characterize the morphology and behavior of various species, undergo evolutionary processes under selective regimes from the hypogean environment (Trajano et al. 2009), or, alternatively, in the lack of selective pressures occurring in external environments. This evidence suggests that continuous adaptation to constant darkness, or the lack of a directional zeitgeber (German for “time-giver”, an environmental stimulus that influenciates the biological clock), can over time lead to the loss of the biological clock function, accompanied by a decrease in features related to light perception, such as ocular structures (Friedrich 2013; Abhilash et al. 2017; Royzenblat et al. 2023). The loss of the circadian cycle is more prevalent in troglobitic species (Hoenen and Gnaspini 1999). On the other hand, animals residing near cave twilight zones demonstrate a notable sensitivity to light, meaning that low-intensity light-dark cycles can still influence the maintenance of rhythms, as observed in Gnaspini et al. (2003). However, it is essential to emphasize that species inhabiting these environments exhibit distinct behaviors from those living on the surface (Oliveira and Ferreira 2014).

The biological clock synchronizes rhythms and is expressed through patterns of locomotion associated with the alternation between activity and rest, habitat exploration, and various interactions, such as agonistic behavior, territory defense, and predator-prey relationships (Volpato and Trajano 2006). Studies have demonstrated the adaptation of the biological clock in cave environments (Friedrich et al. 2011; Friedrich 2013), where zeitgeber variables like light, temperature, and feeding play a significant role in regulating biological rhythms, behavioral patterns, and cellular processes. These factors are possibly linked to the maintenance of peripheral clocks, in which both light and nutrition play crucial roles in regulating the body's peripheral clocks, although their effects occur in distinct ways. Furthermore, absence of regular patterns of light and dark cycle can lead to the evolution of non-circadian clocks, resulting in longer (infradian) (Oda et al. 2000) or shorter (ultradian) rhythms (Pasquali and Sbordoni, 2014). These studies, along with others (*e.g.*, Park et al.

1941; Campbell 1976; Weber et al. 1994; Stringer and Meyer-Rochow 1997; Hoenen and Gnaspini 1999; O-Martínez et al. 2004; Pasquali et al. 2007; Soriano-Morales et al. 2013; Royzenblat et al. 2023), represent significant steps in understanding the adaptations of the biological clock in subterranean environments, although there are still gaps to be explored.

Here we present a review of the existing literature on the rhythmicity of invertebrates in subterranean ecosystems. Four questions were formulated regarding the topic: (i) in which world regions are chronobiological studies regarding cave invertebrates most prominent? (ii) which invertebrate taxa have been the most used to study rhythmicity? Following this, detailed information about the evolutionary characteristics of each taxon documented in subterranean ecosystems was gathered from the examined literature to address other questions: (iii) how does the invertebrates' rhythmic behavior manifest in arrhythmic environments? (iv) is there any correlation between the functional morphology of taxa and the identified biological rhythm pattern?

Materials and methods

Search strategy for collecting data on the rhythmic behaviour

We conducted a systematic literature review following the methodology proposed by Mammola et al. (2022) to compile a list of terrestrial and aquatic invertebrate species inhabiting subterranean systems whose biological rhythms or arrhythmia have been examined and confirmed. Additionally, we created the PRISMA 2020 flow diagram (Liberati et al. 2009; Page et al. 2021), which enhanced the accuracy of representing the direction and approach employed in this systematic review (Figure 1).

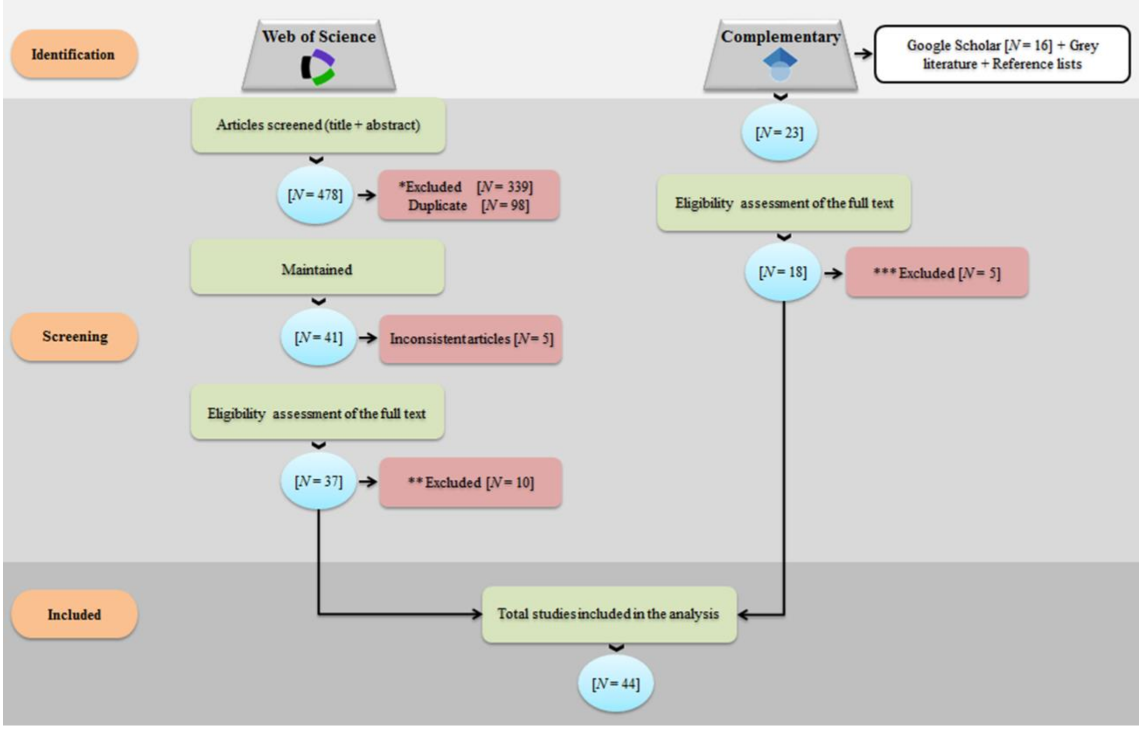

Figure 1. Publications extracted from the WoS database and complementary literature used in this study.

* (i) Vertebrates: fishes, bats, anurans, humans; (ii) Unrelated to rhythm: *e.g.*; (iii) Description of a new species; (iv) Other contents: cultural, excavation, geoarchaeology, geochronology, paleoclimate, etc.
** (i) Not found [*N* = 3]; (ii) Did not identify presence or absence of rhythm [*N* = 3]; (iii) Did not provide tests/conditions related to the presence and/or absence of light [*N* = 2]; (iv) Epigeal species or collected outside natural subterranean cavities [*N* = 2].
*** (i) Did not provide tests related to the presence and/or absence of light [*N* = 2] AND/OR (ii) Epigeal species or collected outside natural subterranean cavities [*N* = 3]

Previous review works that encompassed constant and arrhythmic environments, such as caves, were incorporated: "Biological clocks and visual systems in cave-adapted animals at the dawn of speleogenomics (Friedrich 2013)" and "To be or not to be rhythmic? A review of studies on organisms inhabiting constant environments (Abhilash et al. 2017)." We conducted a systematic review and comparative analysis using the corresponding database of studies involving invertebrates from subterranean habitats that underwent rhythmicity tests. These articles were identified through searches for open-access articles or within the Clarivate Analytics Web of Science (WoS) platform. If not available, they were requested from the corresponding authors.

The searches were conducted in September 2023. The strategy included the use of descriptive terms covering all publication fields, with no language or publication year restrictions, following two stages: "Preliminary Investigation" and "Data Selection and Organization".

Preliminary investigation

A variety of search terms were employed to enhance the literature retrieval process. Based on this initial exploration, search terms were optimized to reduce the number of irrelevant references (Mammola et al. 2022). The use of broad search terms such as "Cave," "Subterranean," "Circadian," "Adaptation," and "Evolution" resulted in a substantial number of scientific articles. For instance, when searching with the term "Circadian," more than 62,000 articles were found. The keyword "Cave" yielded over 37,000 articles, and the term "Subterranean" produced more than 11,000 articles. However, most of these articles did not contain relevant information for this study, as they pertained to topics such as vertebrates, archaeology, climatology, paleontology, among others.

Data selection and organization

Initially, keywords were applied independently: Circadian clock, Circadian rhythm, Rhythm, Light-dark cycle, Chronobiology, Arrhythmic, Locomotor activity, Ultradian, Infradian, Zeitgeber time, Activity rhythms. For each keyword, the word "Cave" was added to the search for a more thorough approach. Subsequently, a screening process was carried out based on titles and abstracts. Articles lacking information about animal biological rhythms, unrelated to invertebrates, or concerning non-subterranean species were excluded. Following this, a thorough analysis of the full texts of the selected references from the screening was conducted to determine their relevance to the scope of this review.

During the data incorporation phase, invertebrates were categorized as terrestrial, aquatic, or semiterrestrial. Subsequently, species were grouped into the following categories based on interpretations from the collected references: i) Taxonomic classification (Phylum, Subphylum, Class, Order, Family, and Species); ii) Ecological-evolutionary classification of organisms according to the Schiner-Racovitza system; iii) Ecological-evolutionary classification of organisms based on their habitat: aquatic, semiterrestrial, and terrestrial; iv) Type of biological rhythm (arrhythmic, circadian, infradian, and/or ultradian) under light-dark cycles (LD), constant light (LL), and/or constant darkness (DD); v) Phototaxis (if tested: negative or positive); vi) Purpose of the study (locomotor activity, cuticular moulting cycle, feeding and foraging stimuli, among others); vii) Geographic regions of the caves where the tested species were collected. Relevant observations regarding the rhythm of the studied species were also included. However, data that showed inconsistencies, such as the absence of information about the biological rhythm when evident, as well as non-cave-dwelling or non-subterranean invertebrate species, were excluded from the analysis.

Expansion of the bibliographic research

A search was conducted on Google Scholar, following the methodology described by Haddaway et al. (2015), using the same keywords listed in the previous section. These additional searches resulted in the inclusion of 16 additional articles in our database. Furthermore, for each of the previously mentioned articles, we thoroughly analyzed the reference lists to ensure comprehensive coverage in the literature review. Finally, a search for unconventional grey literature, not listed in WoS, was conducted, including articles written in languages other than English. All these additional sources (N = 23) were categorized as 'Supplementary' in the database and PRISMA.

Statistical analysis and data representation

Due to the limitation of the sample size, there was no restriction on the temporal analysis, with the exception that data for 2023 covers only up to the end of September.

All analyses were conducted in the R environment, version 4.2.3 (R Development Core Team, 2022), using the 'ggplot2' (Wickham 2016) and 'circlize' (Gu et al. 2014) packages for data visualization.

To create the global Kernel map, QGIS 3.32.3 'Lima' (2023) software was used, where 'shapefiles' and specimen collection data were previously transformed into the WGS84 geographic coordinate system. In order to assess the distribution densities in the collection areas of the studies selected based on eligibility criteria, the Kernel Density analysis technique was employed. A buffer with a radius of 300 kilometers was created around each point to represent the intensity.

A non-parametric Chi-Square test was conducted based on the creation of a contingency table to assess the existence of a dependency between the classification levels as defined by Schiner-Racovitza (trogloxene, troglophile, and troglobite), identified in the database through the eligibility criteria, and the presence or absence of rhythmicity. Furthermore, standardized Pearson residuals, also known as 'z-scores,' were calculated to identify potential outliers in the dataset, given their ability to measure the degree of deviation of a value from the mean in terms of standard deviation (Pearson 1895; McHugh 2013). Subsequently, these residuals were adjusted (intervals > 2.77 or < -2.77) with a correction of the residual limit (cut-off point) and a significance level of $\alpha = 0.0056$, considering the number of cells in the contingency table to ensure that the results are statistically significant (MacDonald and Gardner 2000; Sharpe 2015).

Results

Overview of the sampled literature

In the research conducted in the Web of Science database, 478 studies were identified. Among these, only 9% demonstrated relevance after the analysis of titles and abstracts for the full-text analysis. Furthermore, 23 more articles were included through known literature and references found on Google Scholar and in grey literature. In total, 44 studies met the inclusion criteria (presence or absence of biological rhythms in invertebrates from subterranean habitats) (Figure 1), with 25 studies identified in the works of Friedrich (2013) and Abhilash et al. (2017). The year 2005 recorded the highest number of selected documents (Figure 2B). The compiled dataset exhibited varied taxonomic diversity, covering 45 species, with over 80% of them being terrestrial (Figure 2C), and the order Coleoptera being the most representative (Figure 3). Furthermore, a geographically restricted distribution was observed (Figure 2A).

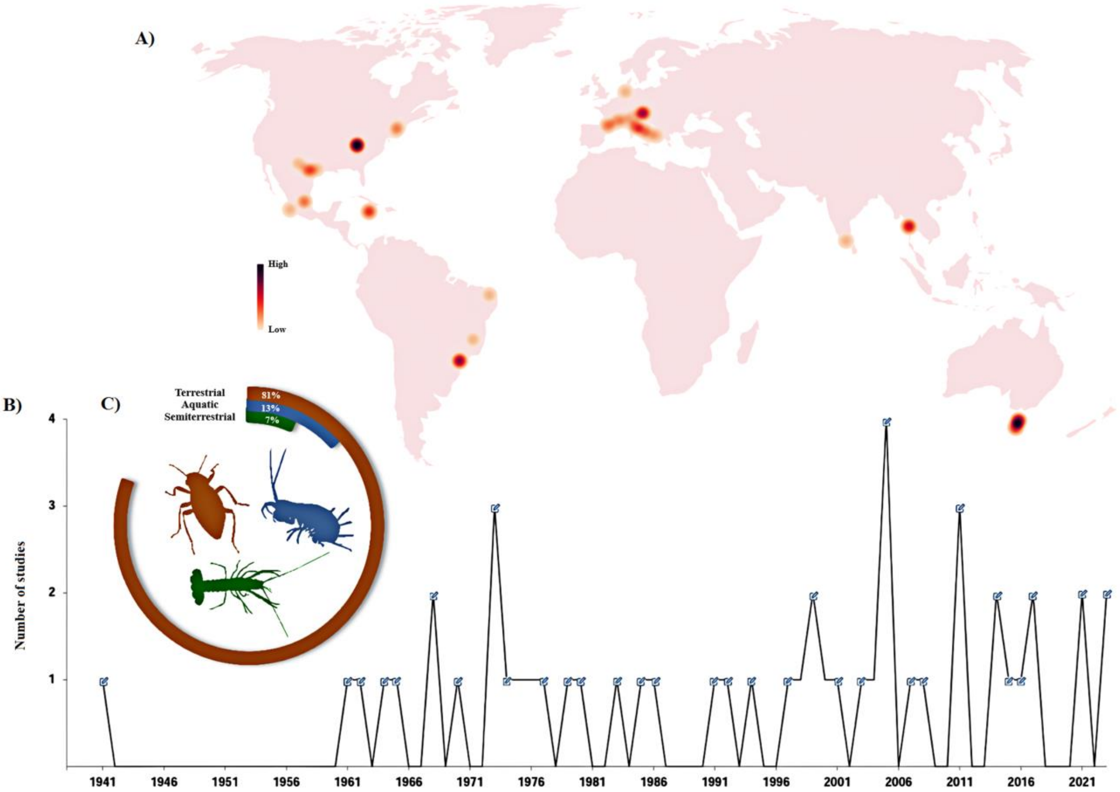

Figure 2. Summary of the Collected Data. In "A", the proportion of the geographic distribution of the sampled studies in relation to the collection of specimens is presented. Colour-coded categories represent the frequency of collection sites for rhythmicity tests with cave invertebrates. The temporal distribution in "B" illustrates the studies selected for analysis. "C" records the occurrence of ecological-evolutionary classes of species based on their environmental type (terrestrial, aquatic, and semi-terrestrial).

Most of the studies were conducted in the United States (25%), followed by Italy (17%), France (14%), Australia (12%), and Brazil (11%). No records of collections and studies on the chronobiology of cave invertebrates in the African continent were identified, as indicated by the sampled data.

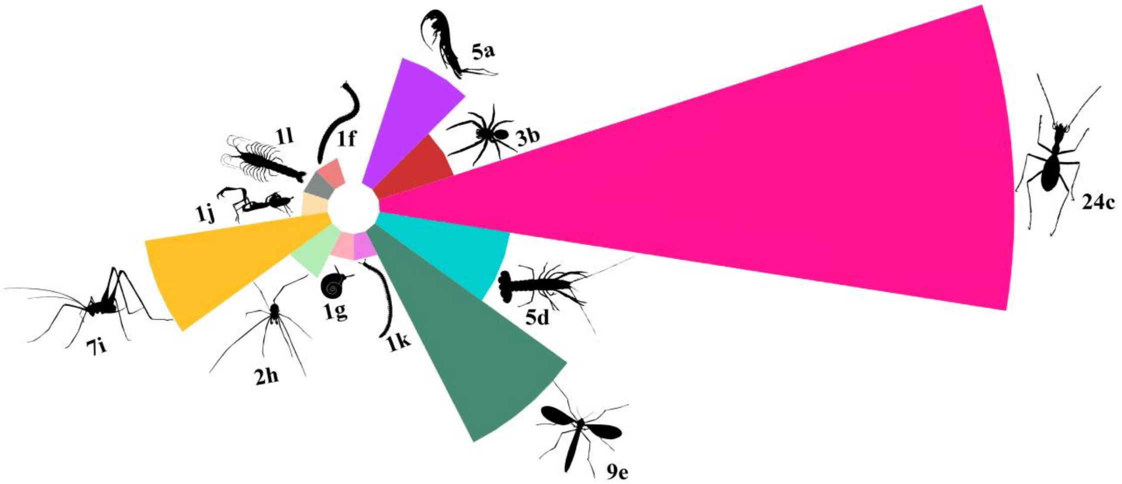

Figure 3. The representativeness of taxon orders in rhythmicity studies. The number next to the silhouette is proportional to the number of times the order appears in the literature selected by eligibility.

a: Amphipoda; b: Araneae; c: Coleoptera; d: Decapoda; e: Diptera; f: Spirostreptida; g: Littorinimorpha; h: Opiliones; i: Orthoptera; j: Squizomida; k: Julida; l: Stygiomysida.

Furthermore, among the studied species, troglobitic and troglophilic organisms were the most representative, totaling 24 species and 16 species, respectively. Only two species [*Goniosoma spelaeum* (Mello-Leitão) and *Anopheles dirus* (Peyton and Harrison)] were classified as trogloxenes.

Types of response regarding classification levels

The analysis indicated a significant dependent relationship between the ecological-evolutionary status of cave invertebrate species and the presence or absence of rhythms under DD and LD conditions (*p*-value: 0.0205 and 0.0062, respectively), providing compelling evidence (Figure 4A, C). However, the LL treatment (*p*-value: 0.0021) revealed a substantial relationship, yet the scarcity of studies investigating this light regime limited the availability of sufficient data to conclusively establish a dependent relationship (Figure 4B).

Among the ecological-evolutionary categories, the "Troglobite" category exhibited the highest dependency between response types (presence or absence of rhythm) under both DD and LD conditions, followed by the "Troglophile" category in the same light regimes. The

"Trogloxene" category showed no statistical differences (Table 1) in any of the three light treatments, possibly due to the low number of animals belonging to this category that were considered. Studies that reported rhythmic and arrhythmic patterns for the same species also showed no difference, considering the absence of information regarding intrinsic conditions of individuals, such as developmental stage (larva or adult) or reproductive status (virgin females, inseminated females, or males), which may be attributed to their infrequent occurrences. Additionally, it was observed that the troglobites had the highest frequency of individuals exhibiting arrhythmia (Table 1). However, for the troglophiles, both in constant light (LL) and in the light-dark cycle (LD), the highest frequency occurred in the presence of biological rhythms, with values higher than expected if there were no association between the variables.

Table 1. The adjusted standardized residuals, considering a significance level of $\alpha = 0.0056$ for p-values, were calculated with intervals greater than 2.77 or less than -2.77.

DD	Troglobite	Troglophile	Trogloxene
Arrythmic	3.4542	-3.2928	-0.5658
p -value	0.00055	0.00099	0.57153
Rhythmic	-4.3693	4.1651	0.7157
p -value	0.00001	0.00003	0.47419
A&R	2.0048	-1.9111	-0.3284
p -value	0.04499	0.05600	0.74263

LL	Troglobite	Troglophile	Trogloxene
Arrythmic	2.1563	-2.1563	-
p -value	0.03106	0.03106	-
Rhythmic	-2.1563	2.1563	-
p -value	0.03106	0.03106	-

LD	Troglobite	Troglophile	Trogloxene
Arrythmic	2.9245	-2.4906	-0.8286
p -value	0.00345	0.01275	0.40736
Rhythmic	-3.57	3.0403	1.0114
p -value	0.00036	0.00236	0.31180
A&R	1.5346	-1.3069	-0.4348
p -value	0.12488	0.19123	0.66372

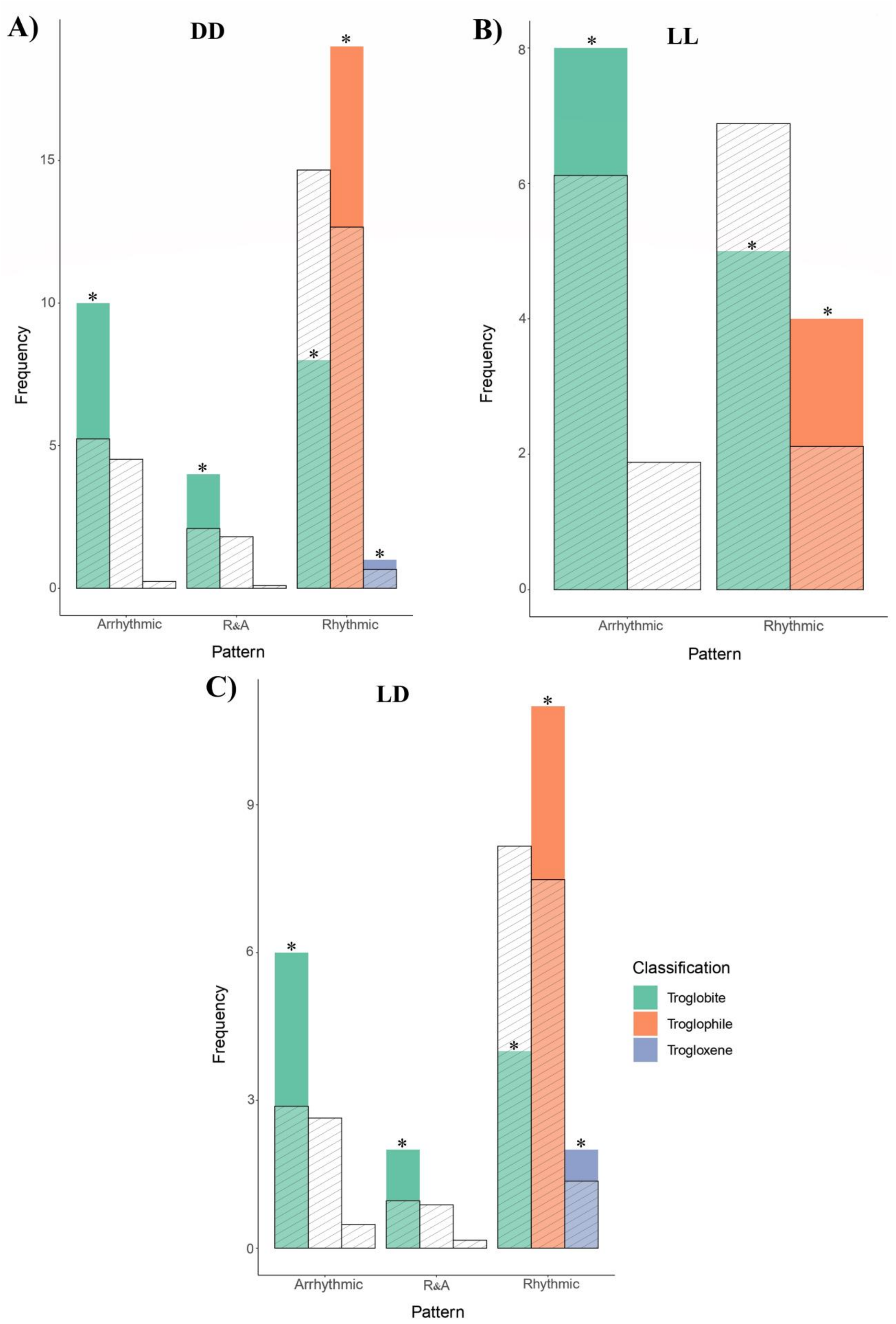

Figure 4. Relationship between the classification levels and the types of responses (arrhythmic and/or rhythmic) of the assessed animals. A) Relative proportion between response types and cavernicolous animal classes (troglobite, troglophile, and trogloxene) under DD treatment. B)

Relative proportion between response types and cavernicolous animal classes under LL treatment. C) Relative proportion between response types and cavernicolous animal classes under LD treatment.

"*" Represents the observed value, and the error bar represents the expected value of the Chi-Square test.

Topics involved in the study of biological rhythms

In the examined studies, the occurrence of rhythms (circadian, ultradian, and infradian), as well as arrhythmia, displayed variation across diverse research topics. These topics included activity period (5%), seasonal behavior (3%), biological and geophysical rhythms (2%), bioluminescence propensity rhythm (3%), flight activity (5%), foraging period (2%), genetic variation in circadian clock genes (2%), the impact of cave lighting on bioluminescence (2%), locomotor activity (63%), motor activity (3%), phototransduction and clock gene expression (2%), reaction to light stimuli (2%), reproductive cycle (2%), respiration (6%), rhythm of bioluminescence (2%), and synchronization of circadian bioluminescence (2%) (Figure 5). Locomotor activity emerged as the most extensively studied, constituting over 60% of the analyzed studies.

Furthermore, it is noteworthy that a considerable number of studies (about 4%) did not provide information on the periodicity or irregularity of rhythms in the studied species (NR) or did not conduct tests (NT). Among these conditions, only a small percentage (4%) of studies were conducted under conditions of constant darkness (DD), while a majority were conducted under constant light (LL) (60% of the studies), and/or light-dark cycles (LD) (over 40%).

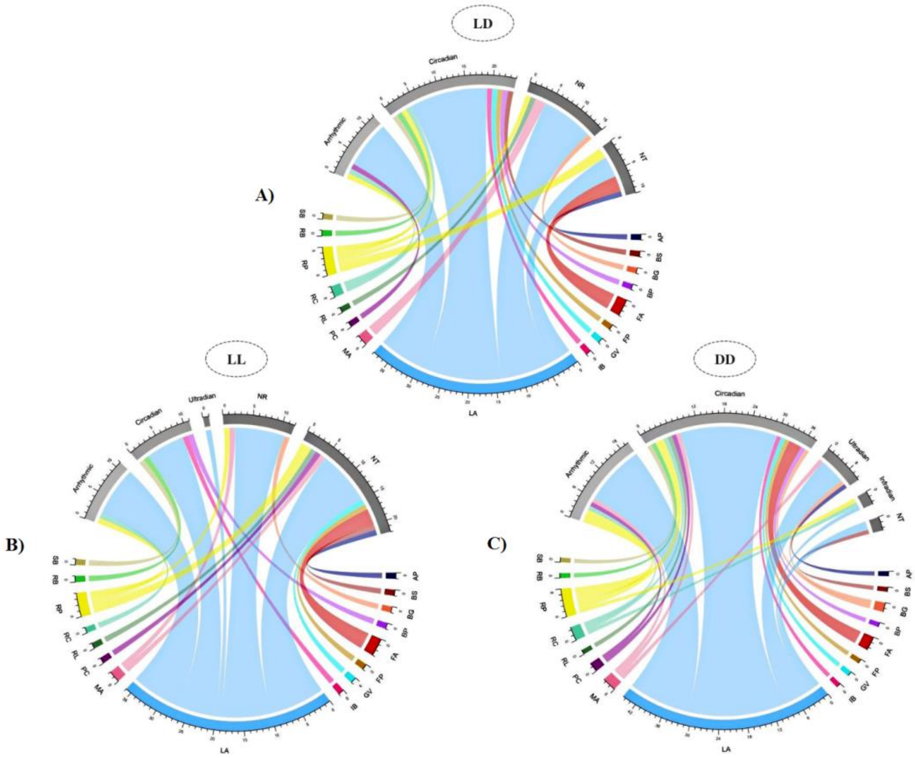

Figure 5. A chord diagram illustrates the interrelationships between the listed research subjects at the bottom and the presence of rhythms (circadian, ultradian, and infradian), arrhythmia, or when untested (NT) or unreported (NR) at the top. A) Under LD treatment (Light-Dark). B) Under constant light (Light-Light). C) Light-dark cycle (Light-Dark).

Research topic abbreviations: AP = Activity Period; BS = Behavior Between Seasons; BG = Biological and Geophysical Rhythms; BP = Bioluminescence Propensity Rhythm; FA = Flight Activity; FP = Foraging Period; GV = Genetic Variation in Circadian Clock Genes; IB = Impact of Cave Lighting on Bioluminescence; LA = Locomotor Activity; MA = Motor Activity; PC = Phototransduction and Clock Gene Expression; RL = Reaction to Light Stimuli; RC = Reproductive Cycle; RP = Respiration; RB = Rhythm of Bioluminescence; SB = Synchronization of Circadian Bioluminescence.

Discussion

In which geographical regions are studies on chronobiology with subterranean invertebrates most prevalent?

A substantial portion of the evaluated studies were conducted in the United States (Figure 2A), with a particular focus on the state of Kentucky (Park et al. 1941; Brown 1961; Reichle et al. 1965; Jegla and Poulson 1968; Studier et al. 1986; Royzenblat et al. 2023). This prominence is likely due to the extensive knowledge and renowned importance of Mammoth Cave National Park, particularly the Mammoth Cave System itself. The cave is not only the most extensive cave system in the world, but it is also known for its high diversity of cave fauna and endemism, among other factors, which make it a highly inviting location for several studies (Barr 1967; Poulson 1992; Friedrich 2011; Culver and Hobbs 2017).

Other countries that stand out for their extensive production in chronobiology studies are Italy and France. The prevalence of studies in these countries, compared to other geographical locations, can be attributed to various factors, including investments in national research and historical aspects, such as the well-established scientific tradition in these regions (Meyer et al. 2015; Zizka et al. 2020). As an example, studies on rhythmicity in animals require the use of electronic devices, lighting systems, and software to improve the efficiency and reliability of data (Halberg 1969; Aschoff 1981). Therefore, they require financial resources and infrastructure, even for *in situ* studies (Stringer and Meyer-Rochow 1997; Gnaspini et al. 2003; Merritt and Clarke 2013; Guadanucci et al. 2015; Espinasa et al. 2016).

In all conducted studies in Australia, there was a consistent focus on the larvae of a specific taxon, *Arachnocampa tasmaniensis* (Ferguson). This focus aimed to investigate the bioluminescence rhythm (Merritt and Clark 2011), bioluminescence propensity rhythm (Merritt et al. 2012; Berry et al. 2017), the synchronization of circadian bioluminescence (Maynard and Merritt 2013), and the impact of artificial lighting on bioluminescence (Merritt and Clarke 2013). It is essential to emphasize that numerous show caves in Australia and Tasmania heavily rely on this species, making it one of the key attractions for visitors. Consequently, research on their biological rhythms is probably influenced by economic motives associated with managing their populations in show caves.

In South America, Brazil, despite being categorized as a developing country, has made a significant contribution to records in the study of chronobiology. Most of these records originate from the Southeastern region, which is the most economically developed region in the country (Arruda and Ferreira 2014). In recent years, Brazil has made substantial advancements in subterranean biology research compared to developed countries (Zampaulo and Simões 2022). This improvement in knowledge regarding subterranean fauna in Brazil can be attributed to the presence of specialized research centers, political initiatives aimed at protecting the Brazilian speleological heritage, and the remarkable diversity of different lithologies with the occurrence of caves.

Only two studies have been conducted on the Asian continent, with a particular focus on India and Thailand. It is noteworthy that over 250 species are strictly associated with subterranean habitats in Southeast Asia (Beron 2015). Despite this, only a few studies have been conducted on the biological rhythms of cave fauna in the continent. Furthermore, a hotspot was recently identified in northern Thailand, hosting more than 30 troglobitic species (Deharveng et al. 2023), indicating a notable upward trend in subterranean biology studies in this region. In India, more research on rhythmicity has been conducted, but with a focus on vertebrates (*e.g.*, Marimuthu et al. 1981; Pradhan et al. 1989), highlighting a certain lack of interest in invertebrates, given that the only work in this region occurred over two decades ago. Studies in cave environments prove to be challenging in many Asian countries, as they require funding, and some regions may have limited resources. Additionally, subtropical caves in East Asia are characterized by high levels of biodiversity and endemism, although most of them are not protected by conservation areas (Li et al. 2022). However, even in highly developed countries like Japan, according to available data, no research addressing the chronobiology of cave invertebrates has been observed.

Finally, the knowledge regarding subterranean fauna in Africa remains limited, primarily relying on data from the 1950s to the 1970s. Therefore, there is a notable gap in studies concerning the chronobiology of cave invertebrates in the existing database (Sharratt et al. 2000; Gunn 2004). This absence is particularly concerning, considering that there are over 280 species of stygobitic crustaceans (restricted to subterranean waters) in Africa (Kayo et al. 2012), among several other cave-restricted taxa. Furthermore, the escalating climate changes and intensification of human activities in the past decades underscore the pressing need for updated knowledge regarding the subterranean species from this continent. Preserving these ecosystems depends on field research, environmental impact assessments, and active conservation strategies. The real risk of subterranean biodiversity loss in Africa requires immediate action (Ferreira et al. 2020; Nanni et al. 2023).

Which taxa of invertebrates have been the most studied in terms of rhythmicity?

The taxon most abundantly represented in the analysed studies was Coleoptera (Figure 3). The significant diversity of epigean beetles extends to subterranean ecosystems, establishing them as one of the insect groups with the highest number of troglobitic representatives globally (Decú and Juberthie 1998). Over 55% of the examined species fall under the troglobite category, enabling substantial comparisons with troglophile and even epigean species. Moreover, these studies offer valuable insights into distinct activity patterns and behavioral strategies between surface and cave-dwelling populations (Barr and Holsinger 1985; Simona et al. 1996; Cardoso 2012). Furthermore, this group typically exhibits a diverse diet (Pellegrini and Ferreira 2011), resulting in low maintenance requirements and ease of breeding in controlled environments when studied (Mammola et al. 2021). A similar rationale can be extended to the orthopteran species to be discussed further on (Allegrucci et al.1987; Hoenen et al. 2001; Pasquali et al. 2005).

Another extensively investigated insect taxon comprises the larva of the dipteran *Arachnocampa tasmaniensis*. In a study conducted by Merritt et al. (2012), it was determined that the lighting employed in a tourist-visited cave did not exert a significant impact on the regularity of bioluminescent displays by the larvae. Additionally, a comparative study between *A. tasmaniensis* and *Arachnocampa flava* (Harrison) (an epigean species) revealed disparities in the regulatory mechanisms of bioluminescence (Berry et al. 2017).

Amphipods constituted 7% of the invertebrates considered in the study (Figure 3), emerging as the most numerous groups within the Malacostraca class concerning the number of species scrutinized for rhythmicity. The selection of this taxon for such investigations was grounded in its remarkable variability in locomotor activity rhythms and its adaptability to a diverse array of habitats and microhabitats (Rossano et al. 2008). Moreover, amphipods display significant morphological diversity and specific adaptations for survival in subterranean environments, including sizable bodies and elongated appendages, albeit with limited migratory abilities (Fišer C et al. 2012; Delić 2017; Ramm 2017). These features render this group highly appealing for chronobiology studies. In their examination of *Niphargus thuringius* (Schellenberg), Galbiati et al. (2023) proposed that this species might serve as a promising model for future investigations to delineate potential distinctions between populations inhabiting surface water environments and those residing in deeper subterranean ecosystems. Still within crustaceans, the decapod species *Orconectes pellucidus* (Tellkampf) has undergone extensive scrutiny due to its association with approximately endogenous 24-hour rhythms and remarkable plasticity in constant environments (Fingerman and Lago 1957; Jegla and Poulson 1970; O-Martínez et al. 2004).

Activity patterns in organisms residing in constant environments offer valuable insights into their biology and the adaptive significance of their circadian rhythms (Miller 1974), as evidenced in the stygiomysid *Spelaeomysis bottazzii* (Caroli) and the diplopods *Blaniulus lichtensteini* (Brölemann) and *Glyphiulus cavernicolus* (Silvestri) (Mead and Gilhodes 1974; Koilraj et al. 2000; Pasquali et al. 2007). Furthermore, for certain arachnids, investigating foraging rhythms and locomotor activity can provide pertinent information about their behavior and adaptations, as observed in non-troglobitic species like *Goniosoma spelaeum*, *Trechona* sp., *Euagrus luteus* (Gertsch), and *Ctenus mitchelli* (Gertsch) (Hoenen and Gnaspini 1999; Gnaspini et al. 2003; Soriano-Morales et al. 2013; Guadanucci et al. 2015). The sole schizomid studied for biological rhythm assessment, *Rowlandius potiguar* (Santos, Ferreira and Buzatto), was chosen as a model for investigating behavioral rhythms due to the limited information available on this taxon, particularly in its cave populations

(Oliveira and Ferreira 2014). Another unique taxon studied was the gastropod *Potamolithus troglobius* (Simone and Moracchioli) (Bichuette and Menna-Barreto 2005), possibly owing to the scarcity of studies on neotropical snails and its inclusion in another work focusing on the behavior and ecological aspects related to this species (Bichuette and Trajano 1999; Bichuette and Trajano 2003).

How does the invertebrates' rhythmic behavior manifest in arrhythmic environments?

The preliminary studies have demonstrated the persistence of organisms with functional biological clocks in cave environments (Brown 1961; Jegla and Poulson 1968; Mead and Gilhodes 1974; Martin and Weber 1985; Koilraj et al. 2000; O-Martínez et al. 2004; Pasquali et al. 2005; Pasquali et al. 2007; Friedrich et al. 2011; Auletta et al. 2017; Galbiati et al. 2023). These biological clocks maintain their regulatory functions, synchronize spatial and temporal rhythms, coordinate metabolic processes (Oda et al. 2000), and govern unrelated cellular mechanisms (Abhilash et al. 2017). These processes suggest that biological clocks remain preserved in cave-dwelling species, playing a crucial role in regulating sleep and rest patterns (Friedrich 2013; Nikhil and Sharma 2017), a pattern also observed in troglomorphic fish (Duboué and Borowsky 2012).

However, the presence of circadian clocks in organisms residing in constant environments prompts questions about their necessity (Abhilash et al. 2017). While there is no definitive conclusion, evidence of temporal organization exists in various species (Campbell 1976; Martin and Weber 1985; O-Martínez et al. 2004; Pasquali et al. 2005; Rossano et al. 2008; Friedrich et al. 2011; Merritt et al. 2012; Soriano-Morales et al. 2013), and its functionality may vary even among closely related species (Bodharamik et al. 2021). It is hypothesized that the circadian clock of organisms in caves operates autonomously in constant conditions, independent of environmental cues (Bodharamik et al. 2021). Specialized habitats, such as subterranean environments, may offer selective advantages, including reduced competition for resources and protection from predation (Bichuette and Menna-Barreto 2005; Galbiati et al. 2023). The threat of predation can influence the forays of stygobitic organisms into surface habitats, making predation by surface-dwelling species a limiting factor for the activity of these specialized organisms.

Therefore, despite stygobitic (troglobitic) species having adapted to highly stable environments, several species still exhibit circadian rhythms. This is attributed to both the internal coordination of their physiological processes and the likelihood that endogenous rhythms evolve slowly over time (Soriano-Morales et al. 2013). However, some studies suggest that circadian components of activity in troglobiotic invertebrates, such as crustaceans, are lost or weakened (Lamprecht and Weber 1992). This raises the possibility that this rhythmicity may not be essential for maintaining internal temporal order. Furthermore, Moran et al. (2014) suggested that the loss of circadian rhythm in metabolism represents an adaptation to energy conservation in oligotrophic environments, such as caves, where there is no need for rhythmic increases in energy expenditure to prepare for daytime activities like foraging, predator avoidance, and locomotor activity.

Locomotor activity plays a pivotal role in organism adaptation. For instance, cave-dwelling individuals of the isopod *Asellus aquaticus* (Linnaeus) showed a reduction in locomotor and metabolic activity, potentially as an adaptation to intense natural selection in an environment with low energy availability. In contrast, the greater variability observed in surface-dwelling *A. aquaticus* may be attributed to more unstable environmental conditions influencing their activity pattern (Fišer Ž et al. 2019). This is further supported by studies examining locomotor patterns under different light conditions (LD, DD, or LL) (Abhilash et al. 2017). However, despite the majority of examined studies (*N* = 32) addressing this topic, only a few encompass all three light regimes, with merely nine studies including them all.

Comparative information on distinct taxa

Among the arachnids, the non-troglomorphic spiders *Euagrus luteus* and *Ctenus mitchelli*, found in the deeper and intermediate zones, were studied under both constant light (DD) and LD (light-dark cycle) conditions. In both cases, a change in the circadian period of their locomotor activities was observed (Soriano-Morales et al. 2013). Furthermore, the troglophilic spider of the genus *Trechona*, subjected to the same conditions, also showed a change in the circadian rhythm of foraging activity. This was evidenced by its constant presence on the web during the LD cycle in the deeper zones, while its population exhibited a higher concentration in the entrance zone, where the circadian rhythm did not seem to be affected (Braga et al. 2011; Guadanucci et al. 2015).

According to Oliveira and Ferreira (2014), the activity patterns of the schizomid *Rowlandius potiguar* were characteristic of an ultradian rhythm, with two notable peaks of activity, one between 10h00 and 16h00 and another between 22h00 and 04h00. The researchers suggested that this type of response plays a fundamental role in coordinating metabolic and behavioral processes, allowing for the efficient distribution of resources over time and space optimization.

For Opiliones, two species were evaluated. The trogloxen *Goniosoma spelaeum* presented a synchronized rhythm when exposed to a light-dark cycle. This suggests that the time dedicated to foraging may be coupled with lighting patterns, serving as a zeitgeber. On the other hand, the troglobitic harvestman *Pachylospeleus strinatii* (Šilhavý), although not tested in a light-dark cycle (LD), showed a circadian cycle with a bimodal characteristic when subjected to constant darkness conditions. These activity patterns include significant movements in search of mates or trophic resources (Hoenen and Gnaspini 1999).

Among the millipedes, the locomotor activity of the blind and depigmented julid diplopod *Blaniulus lichtensteini*, under constant environmental (free-running) condition (DD), is notably irregular although, at times, clear cycles can be observed, oscillating around durations of 12 or 24 hours (Mead and Gilhodes 1974). On the other hand, the troglobitic spirostreptid *Glyphiulus cavernicolus*, demonstrated cyclic pattherns under constant light (LL), constant darkness (DD), and light-dark cycles (LD), with maximum activity during the dark phase. However, it is important to mention that a small fraction of individuals displayed irregular behavior during crawling periods and even under constant illumination (Koilraj et al. 2000).

Among the gastropods, the free-running activity of the troglobitic species *Potamolithus troglobius* under constant light was compared to another species. Both displayed ultradian rhythms, but individual variations, including the presence of circadian and ultradian rhythms, were observed in the cave-dwelling species. Additionally, these variations can be attributed to genetic variability. Furthermore, the movement of this species was less pronounced when compared to the epigean counterpart (Bichuette and Menna-Barreto 2005).

Among insects, in experiments under constant light and light-dark cycles, the troglophilic beetle species of the genus *Laemostenus* [*Laemostenus oblongus* (Dejean), *Laemostenus schreibersi* (Küster), and *Laemostenus terricola* (Herbst)] exhibited rhythmicity. However, under conditions of constant darkness, the troglophilic species *Laemostenus latialis* (Leoni) displayed an ultradian rhythm, distinguishing itself from the other species and from *Rhadine rubra* (Barr), also troglophilic, which showed circadian activity (Wiley 1973; Lamprecht and Weber 1975; Lamprecht and Weber 1977; Lamprecht and Weber 1979; Weber 1980; Martin and Weber 1985; Rusdea 1992; Rusdea 1999; Pasquali and Sbordoni 2014).

Friedrich et al. (2011) documented the gene expression of the circadian clock in the troglobitic species *Ptomaphagus hirtus* (Tellkampf), which was examined under constant darkness (DD). However, Royzenblat et al. (2023) conducted an assessment under conditions of constant darkness and subjected *P. hirtus* to a shifted light-dark cycle (LD), concluding that this species is arrhythmic in terms of its locomotor activity. This result supports the hypothesis that the circadian gene may still be conserved in species specialized for environments with little or no light, such as the aphotic zone (Pasquali et al. 2007; Friedrich 2013; Beale et al. 2016).

The troglobitic carabid beetles *Aphaenops cerberus* (Dieck), *Aphaenops pluto* (Dieck), *Duvalius delphinensis* (Abeille de Perrin), *Duvalius jurecki* (Doder), *Ptomaphagus hirtus*, *Rhadine subterranea* (Van Dyke), *Rhadine tenebrosa* (Barr), and *Speonomus diecki* (Saulcy), with the latter belonging to the Leiodidae family, exhibited arrhythmic behavior for all three treatments (LL, DD, and LD) in the conducted tests (Wiley 1973; Lamprecht and Weber 1975; Weber 1980; Martin and Weber 1985; Faille and Deharveng 2021). Other troglobitic carabid beetles, such as *Sphodropsis ghilianii* (Schaum) and *Typhlochoromus stolzi* (Moczarski), also displayed irregular rhythms when exposed to constant illumination, a pattern not observed in *Duvalius exaratus* (Schaum) and *Laemostenus navaricus* (Vuillefroy), the only ones found to exhibit circadian periodicity under this light regime. In *A Aphaenops orpheus* (Dieck) and some individuals of *L. navaricus*, regular circadian rhythms were observed, while others were arrhythmic under constant darkness conditions. Under a light-dark regime, in addition to *L. navaricus*, the beetles *S. ghilianii* and *T. stolzi* also showed 24-hour rhythms (Lamprecht and Weber 1973; Martin and Weber 1985; Weber et al. 1994).

Stringer and Meyer-Rochow (1997) conducted tests of daytime flight activity in dipterans from the families Scatopsidae, Phoridae, and Milichiidae, which are likely troglophiles. They reported the predominance of circadian rhythms, even in species found in the deeper parts of the cave. This suggests the possibility that these insects use the activity of bats during the night and early morning in the cave as a temporal signal (zeitgeber). On the other hand, the larvae of *Arachnocampa tasmaniensis* were extensively studied compared to other insect species (Merritt and Clark 2011; Merritt et al. 2012; Maynard and Merritt 2013; Merritt and Clarke 2013; Berry et al. 2017) and showed coupling of the circadian clock under all three lighting regimes. Finally, *Anopheles dirus*, a trogloxen species, also exhibited behavioral (flight activity) synchronization in LD and circadian oscilation under DD (Bodharamik et al. 2021).

There are no studies in which cave crickets were subjected to constant light conditions (LL). Among the analyzed articles, only *Strinatia brevipennis* (Chopard) and *Ceuthophilus conicaudus* (Hubbell) were evaluated under a light-dark and constant dark regime, and in both species, the presence of a circadian rhythm in locomotor activity was observed (Campbell

1976; Hoenen et al. 2001; Hoenen 2005), with the former exhibiting low-intensity photophobia (Hoenen and Marques 1998). Studies on Rhaphidophoridae species also showed the same response in constant darkness; however, the ultradian rhythm was also manifested in individuals of *Dolichopoda baccetti* (Capra), *Dolichopoda geniculata* (Costa), and *Hadenoecus subterraneus* (Scudder) (Reichle et al. 1965; Simon 1973; Allegrucci et al. 2005; Pasquali et al. 2005). In both *Dolichopoda* species, variability in the circadian period was observed when compared to populations in an artificial cave (Pasquali et al. 2005).

Among the crustaceans, the troglophilic amphipod *Niphargus puteanus* (Koch), although not showing specific nighttime activity under light-dark conditions (LD), exhibits photonegative behavior. Furthermore, a less pronounced response in constant free-running darkness after a period under light-dark cycles suggests the presence of an ultradian biological clock that regulates its locomotor activity (Blume et al. 1962). An ultradian rhythm with an average period of 16 hours was also identified in the troglobitic *Niphargus poianoi salernianus* (Karaman) tested under constant darkness (DD). However, a clear expression of this rhythm in a light-dark environment (LD) was not observed, although the average period was approximately 23.2 hours. It is plausible that these organisms have an endogenous rhythm since they likely use different signals than light to synchronize their endogenous metabolic processes (Auletta et al. 2017). In a study related to light stimulation in the troglobitic *N. thuringius*, a strong photophobic response was demonstrated, similar to *N. puteanus*, and a circadian rhythm was observed in constant darkness conditions (Galbiati et al. 2023). Rossano et al. (2008) noted that the semi-terrestrial troglophilic amphipod, *Orchestia gammarellus* (Pallas), showed notable plasticity in its locomotor activity, displaying high variability, although it had a weaker definition of circadian rhythm compared to non-cave-dwelling species also evaluated in constant darkness. In contrast to the aforementioned species, the troglobitic *Stygobromus allegheniensis* (Holsinger) did not exhibit circadian rhythm synchronization concerning motor activity. This suggests that the circadian rhythm in this species underwent an initial process of regressive evolution (Espinasa et al. 2016).

Brown (1961) reexamined the activity of the troglobitic crayfish, *Orconectes pellucidus*, which had been previously studied by Park et al. (1941). Previous studies indicated 24-hour and infradian rhythms, with minimal activity levels in the morning and maximum activity in the evening. Subsequently, in the study conducted by Jegla and Poulson (1968), a circadian periodicity in locomotor activity, oxygen consumption, and reproductive cycles (Jegla and Poulson 1970) was identified in some specimens of the same species under controlled constant darkness (DD) condition.

Notably, some individuals of *O. pellucidus* exhibited aperiodic behavior, making the initial work of Park et al. (1941) the first to document arrhythmicity in an obligate subterranean invertebrate. This observation occurred amid photonegative responses and a notably reduced visual system, suggesting that the opsins proteins of these crayfish indeed indicate photosensory functionality, as noted by Friedrich (2013). The free-running period of the circadian rhythm was also observed in the crayfish *Procambarus cavernicola* (Mejía-Ortíz, Hartnoll and Viccon-Pale), with periods of 23.2 hours in DD, 20.1 hours in LL, and 21.5 hours under entrained conditions (O-Martínez et al. 2004).

The anophthalmic troglobitic shrimp *Spelaeomysis bottazzii* displays motor activity with circadian and ultradian periodicities in constant darkness, suggesting an endogenous circadian rhythm (Pasquali et al. 2007).

Is there any correlation between the functional morphology of taxa and the identified biological rhythm pattern?

Throughout their evolution, troglobites typically undergo morphological, physiological, and ethological adaptations to cave environments. According to Friedrich (2013), troglobites can be categorized into three major groups based on the extent of retention of visual systems. These categories include the macrophthalmic (with nearly total retention of visual systems), microphthalmic (with slight to nearly complete loss of the visual system), and anophthalmic species (lacking any trace of a visual system). This classification is essential for understanding trends in the coevolution of biological clocks and visual systems.

The clear convergence in the loss of the circadian rhythm, which is correlated with the absence of eyes, suggests that this may be a predominant evolutionary trajectory for anophthalmic troglobitic species, at least for the beetles (Friedrich 2013). Among the 12 troglobitic beetle species, only two of them, *Sphodropsis ghilianii* and *Typhlochoromus stolzi*, displayed a circadian rhythm in LD. Additionally, *Aphaenops orpheus*, despite being anophthalmic, showed a circadian rhythm under constant darkness conditions (Martin and Weber 1985). However, Friedrich (2011) observed that in *P. hirtus*, although presenting a reduced visual system, it remains functional, and the species possesses a complete circadian clock genetic network. These findings suggest that, as mentioned by Friedrich (2013), even in species without apparent circadian rhythms and lacking eyes, the conservation of the biological clock can be maintained. This allows the regulation of rhythmic behaviors, such as locomotor activity, in response to non-visual cyclic cues, as well as the maintenance of organismal homeostasis. Among the troglophilic species, only *Laemostenus navaricus* and

Duvalius exaratus demonstrated circadian rhythm in the presence of constant light (LL) with arrhythmia in conditions of constant darkness (DD), with the former having some individuals synchronized. These results highlight that some species may be in an evolutionary stage that leads them towards the gradual loss of the clock fuction.

The assessed crickets exhibited ultradian and circadian rhythms with varying amplitudes (Pasquali and Sbordoni 2014). Studies have shown the presence of circadian rhythms in the food-seeking behavior of these crickets, with peaks of nocturnal activity near cave entrances (Campbell 1976; Hoenen 2005; Pasquali et al. 2005). Most of these crickets forage on the surface near cave entrances during the night, indicating an ancestral nocturnal behavior in food seeking and locomotion, as suggested by their preserved lateral eyes (Hubbell and Norton 1978). This combination of a visual system and behavior regulated by the biological clock is a fundamental characteristic of cave cricket biology.

In non-troglobitic dipterans, the circadian rhythm in the regulation of bioluminescence in *A. tasmaniensis* larvae has also been observed, facilitating social synchronization of daily glowing cycles, indicating that variations in the circadian control of physiological functions between cave-dwelling species and those living on the surface may be linked to ecological differences between different habitats (Berry et al. 2017). Additionally, in other dipteran species, the circadian rhythm also plays an ecological role in synchronizing flight activity with the resting period of bats.

However, when it comes to troglobitic millipedes with troglomorphisms, such as elongated bodies, lighter body coloration, and extended femurs and tarsi (Liu 2017), the circadian rhythm in locomotor activity was maintained. Although only two species from different orders were studied (Julida and Spirostreptida), the results were remarkable. This highlights that classical troglomorphisms, by themselves, are not reliable indicators of the degree of circadian reduction in troglobites (Menna-Barreto and Trajano 2015). Furthermore, in crustaceans, the same trend was observed, as most of the evaluated species are troglobitic, with only one species displaying aperiodicity in motor activity. This underscores that troglomorphic traits, such as reduced eyes, pigmentation, and certain behaviors, including cryptobiotic habits and photophobic reactions, often manifest variably in troglobitic populations (Menna-Barreto and Trajano 2015). The same is true for the Littorinimorpha gastropod *Potamolithus troglobius*, which exhibits troglomorphisms as reduced size, weak pigmentation, and diminished visual systems (Simone 2022). This species shows an ultradian rhythm that was lost in an epigean species [*Helisoma trivolvis* (Say)] when their eye peduncles were removed, as shown in Kavalier's work (1981).

It also highlights that the regression of the visual system is not necessarily linked to the regression of the biological clock (Oda et al. 2000).

While most of the arachnids discussed in this review are not obligatory cave dwellers and exhibit very similar rhythms under DD and LD conditions, the troglobitic harvestman *Pachylospeleus strinatii* (Šilhavý 1974) displayed similar outcomes to the others. Hoenen and Gnaspini (1999) found that these troglobitic harvestmen exhibited highly pronounced circadian rhythmicity and demonstrated differences in feeding behavior and light responsiveness compared to surface-dwelling species.

The correlation between troglomorphy and the regression of the biological clock is notably complex (Friedrich 2013). Based on the presented data, it is evident that there is no definitive consensus, and it is not advisable to apply a standardized approach to the evolution of the biological clock and functional elements across all taxonomic groups.

Conclusions

(1) This study emphasizes the necessity to address gaps in chronobiology research by incorporating a wide range of geographic regions and diverse taxonomic groups. The urgency to meet this requirement arises from the limited scope of study areas and the lack of information on tested taxa.

(2) Furthermore, it is essential to incorporate research that investigates the temporal, evolutionary, and ecological processes involved in the decoupling or synchronization with different types of biological clocks (peripherals and central). This is crucial to gain a deeper and more comprehensive understanding of subterranean life adaptations and insights into the functioning of biological rhythms, to deepen our knowledge of the mechanisms behind the regulation of biological rhythms in subterranean environments, especially considering emerging global threats such as climate change and habitat loss. This will help preserve subterranean ecosystems and ensure their resilience in the face of these threats.

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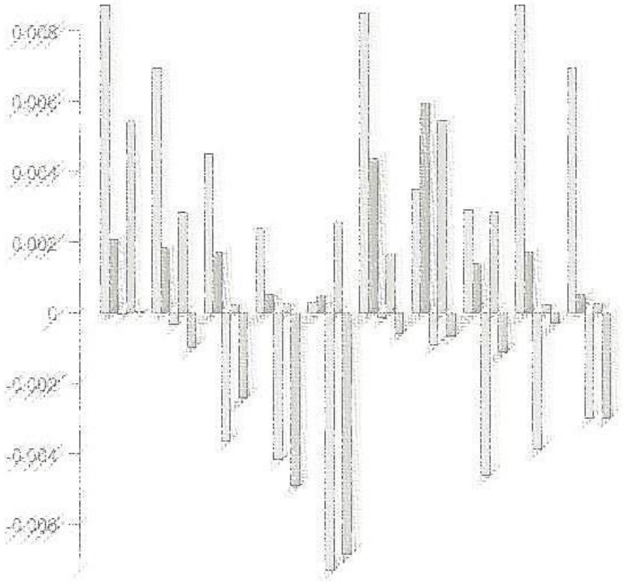

ARTIGO II

DIVERGENT PATTERNS OF LOCOMOTOR ACTIVITY IN CAVE ISOPODS (ONISCIDEA: STYLONISCIDAE) IN NEOTROPICS

ARTIGO II

Este manuscrito foi elaborado em sua versão preliminar, seguindo as diretrizes estabelecidas pela revista *Biological Rhythms*

Divergent Patterns of Locomotor Activity in Cave Isopods (Oniscidea: Styloniscidae) in Neotropics

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Abstract

In cave environments, characterized by stable conditions, including a absence of light-dark cycles and minimal temperature fluctuations, circadian clock mechanisms persist across several species. However, species more adapted to these environments may exhibit arrhythmia in circadian rhythm related to locomotion. This study investigates the potential loss of rhythm due to convergent evolution in five species of semi-aquatic troglobitic isopods (Crustacea: Styloniscidae), with a focus on its impact on locomotor activity. The hypothesis was formulated that these species exhibit aperiodic patterns in their locomotor activity. The sampled isopods were subjected to three treatments: constant darkness (DD), constant light (LL), and light-dark cycles (LD 12:12), totalizing 1656 hours (576 hours in LL and DD, 504 hours in LD). Circadian rhythm analysis involved the Sokolove and Bushell periodogram chi-square test, Hurst coefficient calculation, intermediate stability (IS), and activity difference for each species. All species predominantly showed an infradian rhythm for DD and LL. Light influenced rhythms under LD, indicating synchronization with circadian rhythms. *I. iuiuensis*, *Xangoniscus* sp.2, and *X. aganju* displayed diurnal activity, while *Xangoniscus* sp.1 and *X. santinhoi* exhibited nocturnal activity. The Hurst coefficient indicated rhythm persistence, with higher LD variability. In terms of Intermediate Stability (IS), LD showed higher values, signifying synchronized rhythm across species. Significant variations were observed among individuals of the same species in the three conditions. Contrary to the initial

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hypothesis, all species displayed circadian rhythms under light-dark conditions. Analyzing circadian activity is vital for obtaining essential insights into how organisms adapt to non-cyclical environments, emphasizing the need to explore the underlying mechanisms involved.

Keywords: Cave, biological clock, circadian rhythm, invertebrates, locomotor activity.

INTRODUCTION

Biological rhythm can be defined as a process that exhibits periodic oscillations, and its maintenance in a stable environment can unveil intrinsic elements that drive biological rhythmicity, commonly referred to as circadian clocks (Marques and Menna-Barreto, 2003). Thus, the analysis of various rhythms underscores the pivotal role of circadian clocks, typically characterized by a period of approximately 24 hours, in coordinating endogenous processes (Nikhil and Sharma, 2017). The light-dark cycle (LD) stands out as the primary external stimulus that profoundly influences the behavior of the majority of species (Oda *et al*., 2000), alongside factors such as temperature, humidity (Trajano *et al*., 2009), and feeding stimuli (Gnaspini *et al*., 2003).While it may seem intuitive that the circadian clock could lose function in constant environments, such as caves, owing to the absence of regulatory environmental cues (zeitgebers) like light-dark cycles, temperature variations, oligotrophy (Culver and Pipan, 2009; Beale *et al*., 2016), and the consistent quality of water (Poulson and White, 1969), the biological clock mechanism may persist due to its inherent benefits, serving as a physiological preparation for what lies ahead (Beale and Whitmore, 2016).

The creeping locomotor activity and molecular rhythms of the circadian clock are observed in several species inhabiting caves, indicating that the clock remains intact even in seemingly rhythmless environments, albeit with some variability in this process (Lamprecht and Weber, 1975; Martin and Weber, 1985; Rusdea, 1999; O-Martínez *et al*., 2004; Friedrich, 2011; Duboué and Borowsky 2012; Pasquali and Sbordoni, 2014). However, certain species strongly associated with these habitats (troglobites and stygobites) may exhibit locomotion-linked arrhythmia (Weber, 1980; Trajano *et al*., 2009; Cavallari *et al*., 2011; Royzenblat *et al*., 2023).

Despite belonging to distinct evolutionary lineages, cave-restricted species demonstrate striking convergent evolution in various morphological and physiological aspects. This convergence encompasses the development of elongated appendages, reduction, or complete absence of visual structures and pigmentation, decreased metabolism, and specialization of sensory systems (Trajano *et al*., 2009; Friedrich, 2011; Pasquali and Sbordoni, 2014). These characteristics are commonly referred to as troglomorphisms and may vary among different groups of organisms (Christiansen, 1962).Isopods emerge as one of the most prominent taxa in subterranean environments, largely attributed to favorable environmental conditions such as high humidity, even in regions with semi-arid climates, substrate heterogeneity, and the absence of specialized predators (Fernandes *et al*., 2019). Troglomorphisms, characterized by the absence of pigmentation and eyes, are frequently observed in isopods (Campos-Filho *et al*., 2014; Reboleira *et al*., 2015).

The isopods of the suborder Oniscidea primarily inhabit terrestrial environments, although some may exhibit amphibious habits, notably members of the family Styloniscidae (Souza *et al*., 2015; Bastos-Pereira *et al*., 2017). Styloniscidae is one of the most emblematic families, boasting over 30 troglobitic species (Campos-Filho *et al*., 2022a; 2022b; Cardoso *et al*., 2022; Cardoso and Ferreira, 2023). This taxon serves as a notable example of the evolutionary transition between aquatic and terrestrial environments. The successful colonization of terrestrial habitats by these organisms is attributed to a comprehensive range of physiological and morphological adaptations (Broly *et al*., 2013; Sfenthourakis and Taiti, 2015; Bastos-Pereira *et al*., 2017). Consequently, this group emerges as a crucial model for chronobiology studies, offering insights into how evolutionary traits may have influenced their periodicity.However, there is a notable lack of research addressing the rhythmic behavior of isopods from subterranean ecosystems. This gap underscores the urgent need for in-depth investigations in this group, as, despite its representativeness, it remains relatively unexplored concerning behavioral and chronobiological patterns (Cloudsley-Thompson, 1952; Cloudsley-Thompson, 1956; Warburg, 1968; Hariyama *et al*., 2001; Silva *et al*., 2018).This study aimed to investigate the potential loss of rhythmic elements in cave isopods as a result of convergent evolution in subterranean environments. Specifically, we explored whether the potential absence of rhythmicity influences locomotor activity patterns in troglobitic species of Styloniscidae. Thus, we formulated a hypothesis proposing that different semi-aquatic isopod species belonging to the two subfamilies of Styloniscidae (Styloniscinae and Iuiuniscinae) exhibit aperiodic patterns in their locomotor activity. The irregularity in activity patterns is suggested to be a direct consequence of the absence of light regimes in caves, such as the light-dark cycle, and reduced thermal variability, both of which are essential environmental factors for the regulation of biological rhythms (Beale *et al*., 2016).

MATERIALS AND METHODS

Species collection and *ex-situ* breeding

In March 2023, adult specimens of the troglobitic species *Xangoniscus santinhoi* Cardoso, Bastos-Pereira, Souza, and Ferreira, 2020 and *Xangoniscus* sp.1 were sampled in caves of Minas Gerais state (southwestern Brazil). In September of the same year, collections were carried out in Bahia state (northeastern Brazil), targeting adults of the troglobitic species *Xangoniscus aganju* Campos-Filho, Araujo, and Taiti, 2014, *Xangoniscus* sp.2, and *Iuiuniscus iuiuensis* Souza, Ferreira, and Senna, 2015, as detailed in Table 1.

Table 1. Species of troglobitic Styloniscidae used in the laboratory for assessing rhythmicity and their occurrence locations for the scope of this study.

Species	**Cave**	**Locality**	**Latitude**	**Longitude**
Xangoniscus sp.1	Lapa da Lagoinha III	Montes Claros, MG	-16,6771	-43,8723
Xangoniscus santinhoi	Lapa D'Água do Zezé	Itacarambi, MG	-15,0067	-44,1170
Xangoniscus sp.2	Gruta do Padre	Santana, BA	-13,2162	-44,0650
Xangoniscus aganju	Pedro Cassiano	Carinhanha, BA	-13,7982	-43,9136
Iuiuniscus iuiuensis	Lapa do Baixão	Iuiú, BA	-13,5648	-43,6237

All species are classified as semiaquatic (amphibious) (Fig. 1), with those of the genus *Xangoniscus* belonging to the subfamily Styloniscinae. *Xangoniscus* sp.1 was sampled from puddles formed by the water table. *X. santinhoi* was collected from drainage areas, likely resulting from the overflow of the water table. Individuals of *Xangoniscus* sp.2 were discovered in various-sized water puddles scattered throughout the cave, away from the entrance. *X. aganju* was sampled from the intermittent stream flowing through the cave. Only *X. santinhoi* had individuals also found near the cave entrance; the others were exclusively found in deeper zones. *I. iuiuensis* stands out as the sole representative of the subfamily Iuiuniscinae utilized in this study. This species adopts a unique ecological-evolutionary strategy: the construction of shelters using available cave sediment (Souza *et al.*, 2015). Individuals were primarily sampled within water bodies, in regions far from the entrance, within the aphotic zone.

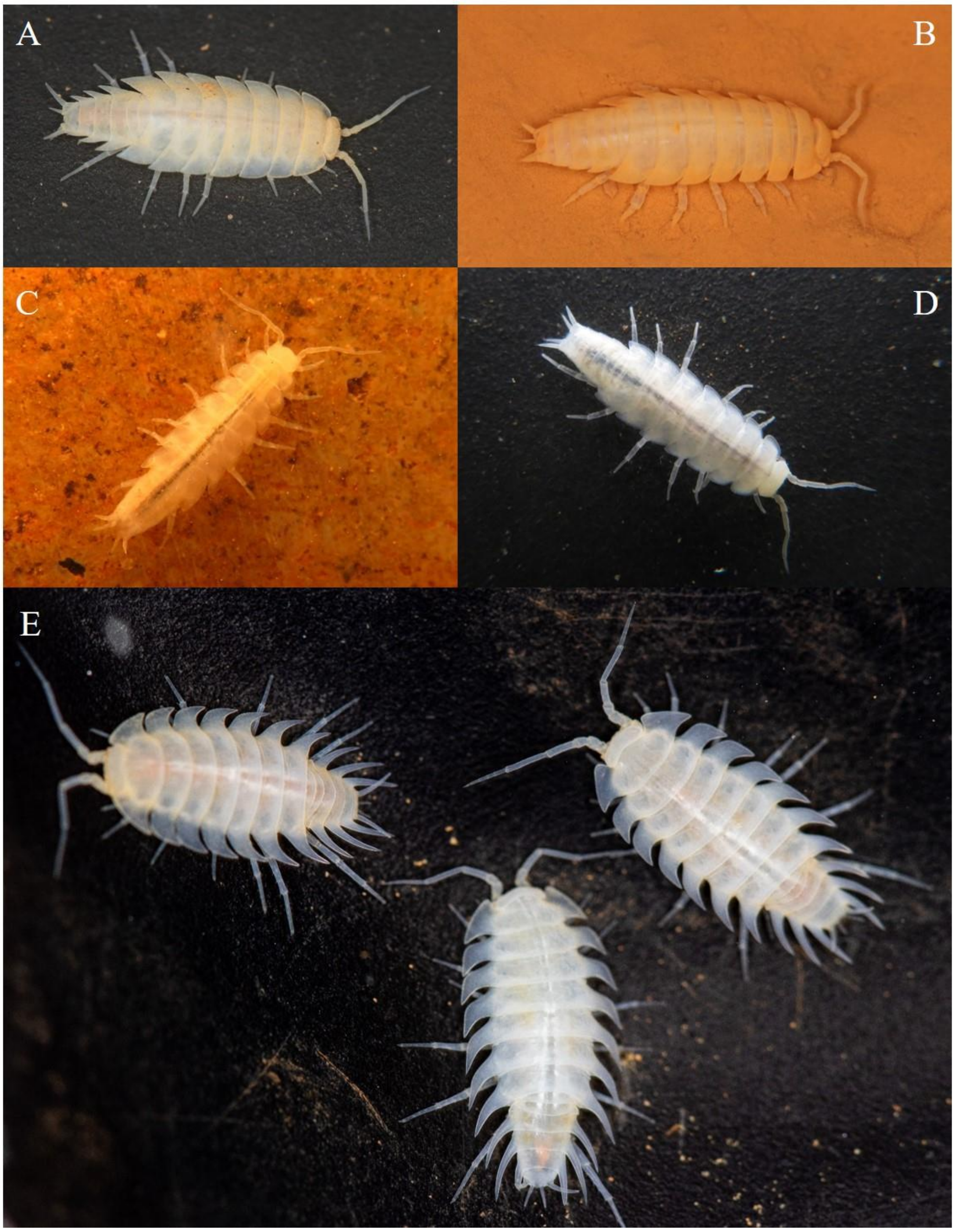

Fig.1. Photographs depicting specimens of the sampled Isopod species for this study. In (A) *Xangoniscus aganju*, (B) *Xangoniscus* sp.2, (C) *Xangoniscus* sp.1, (D) *Xangoniscus santinhoi* and (E) *Iuiuniscus iuiuensis*.

The collections were carried out using aquarium nets No. 4, with the assistance of a soft brush. Ten individuals of each species were captured and transferred to 5L and 10L milk jugs filled with water from the cave itself. Upon reaching the accommodations, still during the field trip, the jugs were sealed with aluminum foil and equipped with an aquarium oxygen aerator. Whenever feasible, the rooms utilized during the collections were climate-controlled to mimic the average temperature of the caves (22ºC ~ 24ºC). These animals were transported in styrofoam boxes to mitigate sudden temperature fluctuations.In the laboratory, the animals were housed in individualized aquariums by species (Fig. 3A) (50 x 35 x 25 cm), located in a darkened, climate-controlled room at the Center for Studies in Subterranean Biology, Federal University of Lavras (UFLA), Minas Gerais, Brazil. The individuals underwent a 15-days acclimation period in complete darkness, during which no data collection occurred.without data collection during this period.

Throughout the tests, individuals were maintained in constant darkness within a climate-controlled room, with temperature settings adjusted to replicate conditions observed in their natural habitat. This was accomplished by monitoring the room's temperature using a digital thermohygrometer, ensuring an average temperature of 23°C (standard deviation = 1°C). As for feeding, flake fish food was provided once a week.

Collection sites

The species were sampled in caves belonging to the largest limestone geological formation in South America, the Bambuí Group (Auler, 2004) (Fig. 3).

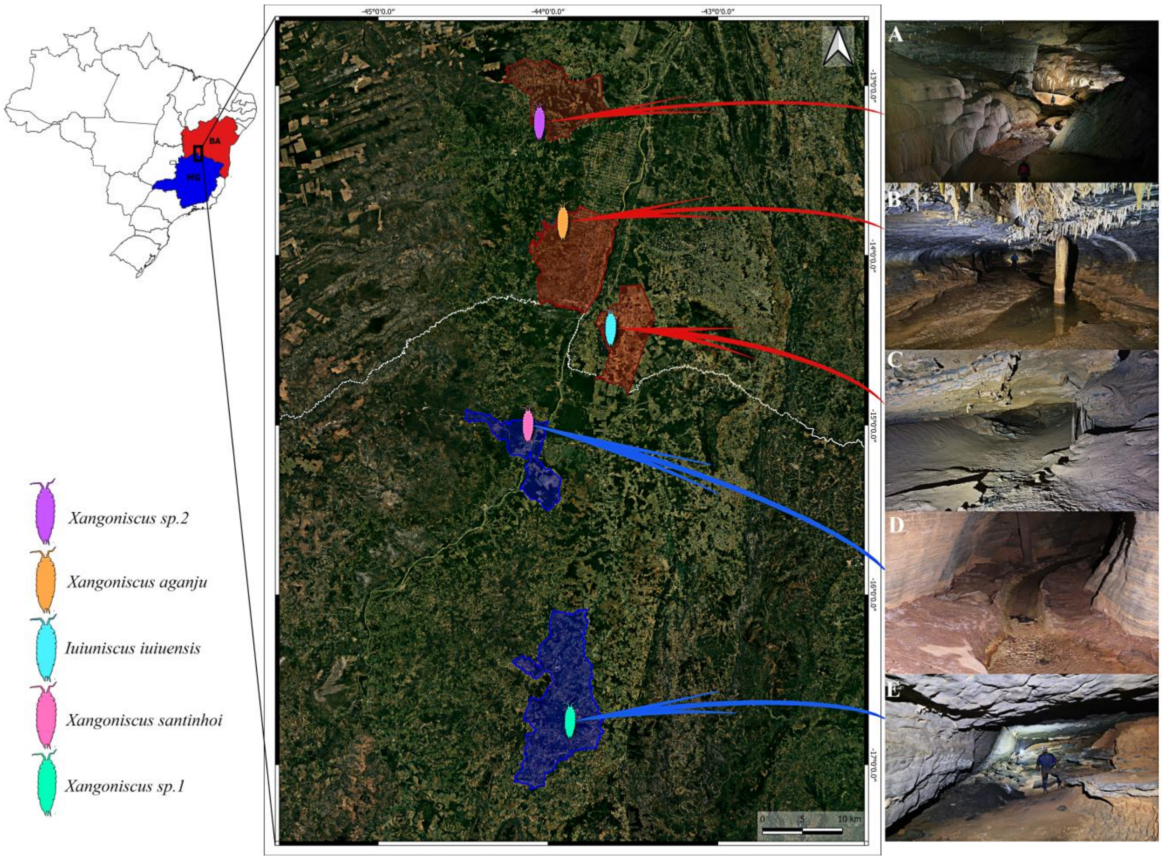

Fig.2. Location of the caves where the collections were conducted. (A) Gruta do Padre cave, (B) Pedro Cassiano cave, (C) Lapa do Baixão cave, (D) Lapa D'Água do Zezé cave and (E) Lapa da Lagoinha III cave. Colored areas (in blue and red) represent the municipalities were the caves are located.

The cave Gruta do Padre, located in Santana, located at the Santa Maria da Vitória municipality, southwestern Bahia state, is situated at the transition between the Caatinga biome and the Seasonal Dry Forest (Cole, 1960). The region has a tropical climate, characterized by dry winters and rainy summers (Köppen-Geiger) with an annual average temperature of 23ºC. Gruta do Padre cave is considered an integral part of the largest subterranean hydrological system in Brazil, which encompasses an extensive underground stretch of the Santo Antônio River (Auler *et al*., 2019). The cave is the fifth largest in the country, with a linear extension of 16,400 meters.

The Pedro Cassiano cave is located in the karst area of Carinhanha, southwestern Bahia, inserted in the middle of the São Francisco River basin. According to the Köppen-Geiger classification, it has an annual precipitation of around 640 mm and an annual average temperature of 23.7ºC, with native vegetation characteristic of the Caatinga biome, interspersed with the Cerrado (Brazilian Savannah) (Cole, 1960; Alvares *et al*., 2013). The cave has a linear extension of about 2,660 meters.

The Lapa do Baixão cave is inserted in the northern part of the large carbonate massif known as the "Serra de Iuiú," in the municipality of Iuiú, the southwest region of the state of Bahia. The area is part of the Caatinga biome (Apgaua *et al*., 2014), characterized by predominantly seasonally dry tropical forests and a hot semi-arid climate, with an average temperature of 24ºC and an annual average precipitation of 788 mm, according to the Köppen-Geiger climate classification (Alvares *et al*., 2013). The cave is estimated to have a length of about 550 meters.

The Lapa D'Água do Zezé cave is located in the northern part of the large carbonate massif in the municipality of Itacarambi, northern region of the state of Minas Gerais. The cave is situated in the transition between two phytogeographical zones: the Caatinga and the Cerrado, a dry climatic region (Cole, 1960) with an average temperature of 24.4ºC and an annual average precipitation of 954 mm (Köppen-Geiger) (Werneck, 2011; Apgaua *et al*., 2014). The cave extends for approximately 300 meters, displaying a labyrinthine morphology.

Finally, the Lapa da Lagoinha III cave, located in Montes Claros municipality, northern Minas Gerais state, is inserted in the Cerrado biomepresents an annual average temperature of 24.2ºC and an annual precipitation of 1,082 mm (Souza *et al*., 2020). The cave has a linear development of 1,087 meters.

Experimental design to record locomotor activity

To assess the activity and rest rhythms of each species, individuals were individually transferred to dark containers (11.5 cm x 6 cm) containing up to five centimeters of water. This was done to contrast with the animals' whitish color, allowing better efficiency in image capture and motion detection through a webcam. Each aquarium contained a pair of containers that were placed inside aquariums with the same dimensions where the specimens were kept (Fig. 3B).

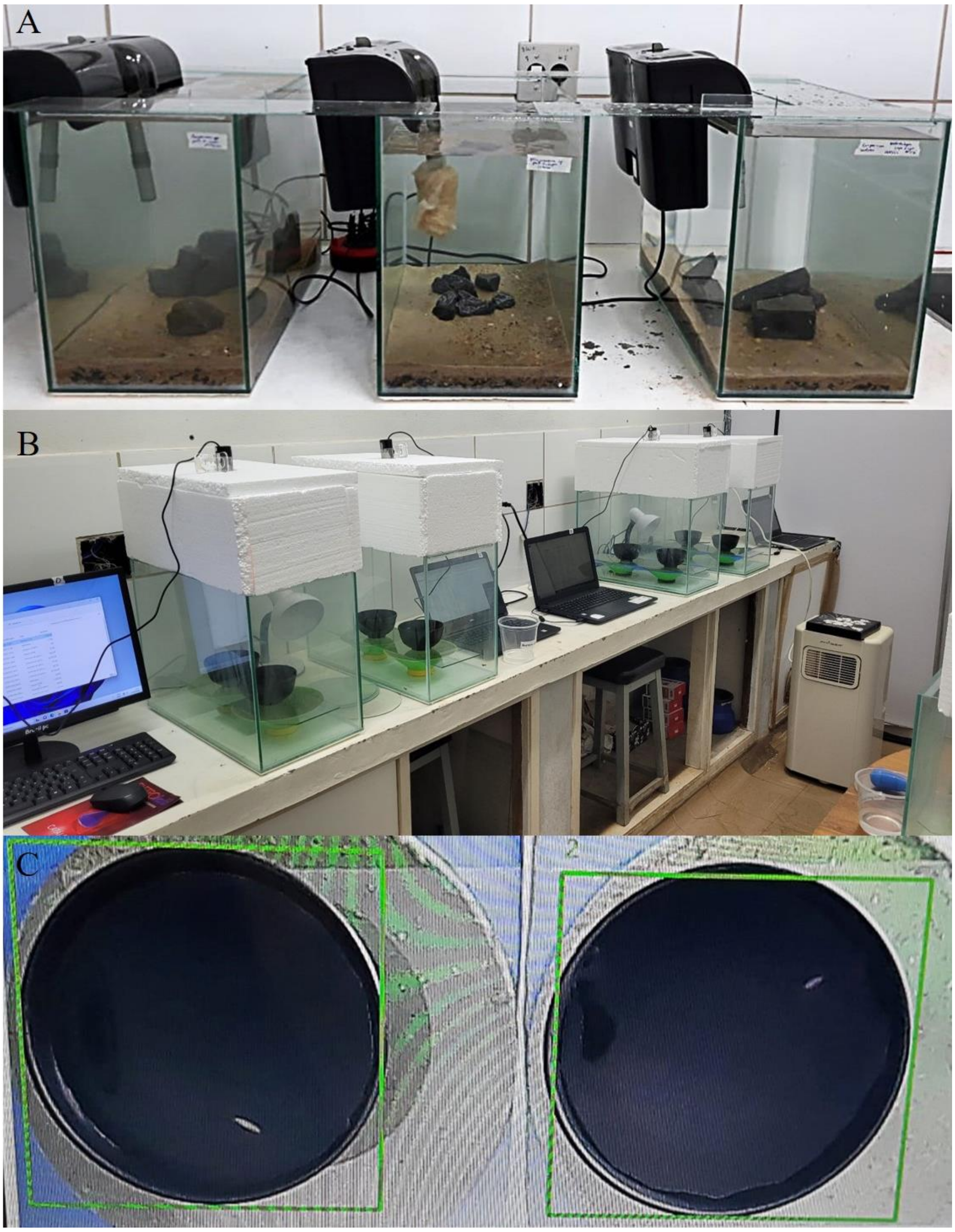

Fig.3. Maintenance and data collection. (A) Aquariums where the specimens were kept; (B) Structuring of recordings under different lighting conditions and (C) Recording the movement

of isopods in dark containers.

Each individual underwent three distinct treatments. The first treatment involved constant darkness conditions (Dark-Dark/DD), maintained under red light emitted by cool lamps positioned 10 cm from the aquariums. Prior to this treatment, specimens were tested to confirm their inability to detect red light. The second treatment consisted of constant light conditions (Light-Light/LL), with an approximate light intensity of 150 lux provided by ceiling-mounted tube lamps. The third treatment allowed for the alternation between light and dark phases (Light-Dark/LD), each lasting 12 hours, as outlined by Sharkey *et al*. (2017). The order of each treatment was conducted randomly. No interventions were made with the animals during filming, and no feeding occurred throughout the duration of the experiments. The locomotor activity patterns of animals were recorded using a webcam and the Aoni ANC HD Lens camera (8.0 megapixels), and analyzed using the open-source software "Arthropod Motion," developed by Voxar at UFPE (https://voxarlabs.cin.ufpe.br/). This study is part of a larger project funded by FAPEMIG (CRA APQ 02822-17), allowing paired sampling where two species were filmed and recorded simultaneously, while locomotor activity was monitored individually. The system detected all movements of the specimens within the water-filled container (Fig. 3C), meticulously recording each body movement and generating concurrent activity-related data as in other studies (Hoenen, 2005; Refinetti, 2008; Friedrich, 2013; Vaze and Sharma, 2013; Pasquali and Sbordoni, 2014). Tests were conducted with the capacity for 12 specimens per session, utilizing six computers equipped with corresponding webcams. The LD (12:12) test was carried out using an analog timer programmed to regulate the room's white and red lamps, alternating every 12 hours.

Prior to conducting the tests, the isopods were acclimated for three days under the same light regime that would be utilized during the subsequent experimental procedures. For instance, the animals underwent three days of exposure to the LL treatment before being observed for an additional three days under the same conditions. Following three consecutive days of observation, the organisms were then subjected to the DD free-running condition, and the process continued accordingly.

Recordings for all species were carried out over a span of eight months, capturing activities at intervals of every tenth of a second. Subsequently, this data was converted into minutes for the duration of the experiment, which lasted three days in total. This amounted to a cumulative recording period of 69 non-consecutive days or 1656 hours (576 hours in LL and DD, 504 hours in LD). To avoid potential conflicts related to autocorrelation or pseudoreplication in the datasets, the assessment of individuals was conducted only once. The number of sessions ranged between 6 and 10 individuals per species (Table 2), depending on the availability of organisms sampled in the field.

Table 2. Number of individuals per species tested for each treatment.

Species	**Specimen Qty**		
	LL	**DD**	**LD**
Xangoniscus santinhoi	10	10	10
Xangoniscus sp. 1	10	9	10
Xangoniscus sp. 2	10	8	10
Xangoniscus aganju	10	6	6
Iuiuniscus iuiuensis	8	7	8

Data analysis

The analysis of the isopods' activity data recorded over three days was conducted using the chronobiology free software "El Temps©" 1.276, developed by Prof. Antoni Díez-Noguera from the University of Barcelona (http://www.el-temps.com/features.htm). Locomotor activity patterns were examined based on the data generated by the software. To assess the synchronization of circadian rhythms in the time series, the Sokolove and Bushell (1978) chi-square periodogram test was employed. This statistical tool evaluates the significance of peaks, providing valuable insights into the presence of relevant periodicity and aiding in the identification of rhythmic patterns or arrhythmia (Sokolove and Bushell, 1978). Additionally, boxplots were generated to summarize the main periods of activity observed for each species.The conversion of numerical data from tenths of a second to minute-by-minute intervals, as well as the computation of locomotor activity, was executed using Matlab R2021a software, developed by Mathworks in Sydney, Australia. Additionally, within the same software platform, calculations for the Hurst coefficient (H) utilizing three distinct approaches, intermediate stability (IS), and the variation in activity for each species were also conducted.The Hurst coefficient was utilized to assess the scale invariance in the locomotor activity patterns of the species under various experimental light conditions. Three distinct approaches were employed: the first (H) involved evaluating the time series over the entire three-day period; the second (Hi) focused on observing the activity patterns on the initial day without acclimation in space (*i.e.*, within the black container); the final analysis (Hf) pertained to the last day and aimed to evaluate the adaptation of individuals of each species.The parameters evaluated by the coefficient encompass trend ($H > 0.5$), reversal ($H < 0.5$), and random behavior ($H = 0.5$). As the coefficient approaches 1, the trend becomes more apparent, while values closer to 0 indicate a stronger tendency to revert to the mean (Chiesa et al., 2010). The calculation of the Hurst coefficient ($1 > H > 0$) relied on the initial value (r1) of the autocorrelation function, utilizing the formula as observed in the study by Eke et al. (2002):

$$H = \frac{\log_2(r_1+1)+1}{2}$$

This expression relies on the initial value (r1) of the autocorrelation function and entails the utilization of the logarithm with a base of 2 (log2).

The calculation of the synchronization level, or intermediate stability (IS), was executed to evaluate the robustness of locomotor activity coupling concerning the three applied treatments (LL, DD, and LD). This analysis facilitated the observation of rhythm stability behavior across days and the comparison of individual profiles (Witting *et al*., 1990). The normalized value of the chi-square periodogram is represented as follows:

$$IS = \frac{n\sum_{h=1}^{p} (\overline{X}_h - \overline{X})^2}{p\sum_{i=1}^{n} (X_i - \overline{X})^2}$$

Where:

- *N* represents the total data;

- *p* denotes the number of daily data points;

- *X* is the mean of all days;

- *Xh* is the hourly mean;

- *Xi* are the individual observations.

This calculation offers a more precise analysis of the dynamics of locomotor activity over time (Galasso *et al*., 2019). Furthermore, an assessment of the disparity in activity among all species exposed to the LD treatment was performed. This involved calculating the average number of movement points during both the dark and light periods, and subsequently determining the difference between these two periods. An analysis of variance (ANOVA) was conducted to investigate the performance means associated with Hurst and IS calculations. Subsequently, post-ANOVA comparisons were carried out using the Tukey test. In instances where the assumptions of variance homogeneity, as verified by the Levene test prior to ANOVA, were not met, data underwent transformation using the logarithmic function (Wang *et al*., 2016). If homoscedasticity was not achieved even after transformation, the non-parametric Kruskal-Wallis test was employed, with correction using the Bonferroni method for Dunn's post hoc test (Nwobi and Akanno, 2021). The significance level for all analyses was set at p = 0.05. Graphical visualizations were generated using R software version 4.2.3 (R Core Team, 2023).

RESULTS

Rhythmic identification and activity period

Based on the average calculated through Sokolove and Bushell's (1978) periodogram analysis, it was observed that all species predominantly exhibited an infradian rhythm (greater than 28 hours) and a weak circadian rhythm during the constant dark period (DD). However, Xangoniscus sp.2 displayed a less pronounced infradian period, indicating a more circadian behavior when exposed to constant light (LL), while the other species maintained rhythmicity with a longer period.

Light was demonstrated to influence the rhythm of the species when subjected to light-dark conditions (LD) (Fig. 4), indicating synchronization to this stimulus (zeitgeber). However, for LL and DD treatments, no consistent patterns were observed in the dynamics of rhythms concerning entrainment.

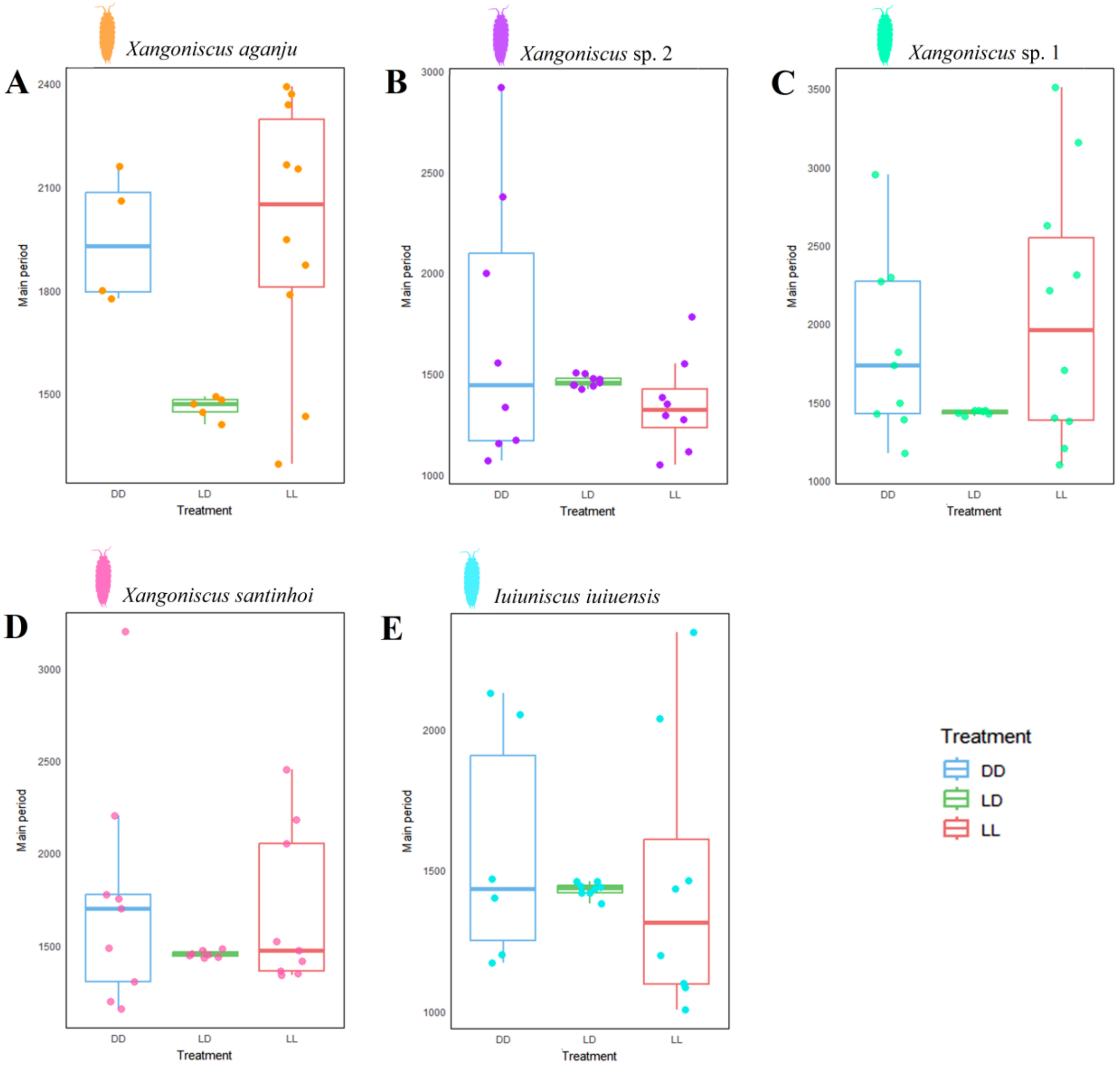

Fig. 4. Representation of synchronization to the circadian rhythm based on the main period for the species. Error bars indicate the variability of the data within each species, while the points represent the observed values from periodograms.

Ultradian behavior was also identified in some individuals of *Xangoniscus* during the constant dark period (DD), while *Xangoniscus* sp.1 (10%) and *Xangoniscus* sp.2 (20%) also manifested it during exposure to constant light (LL).

The species *I. iuiuensis*, *Xangoniscus* sp. 2, and *X. aganju* exhibited diurnal activity in all individuals (Fig. 5), whereas, in the analysis of activity differences, pronounced nocturnal activity was observed for *Xangoniscus* sp. 1 and *X. santinhoi*.

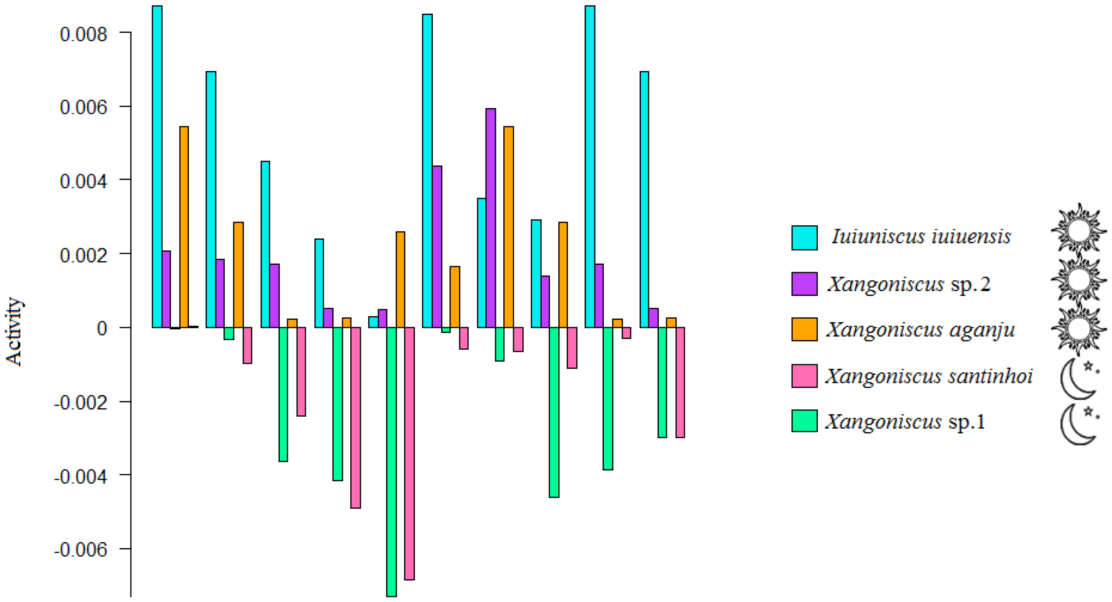

Fig. 5. Difference in laboratory in response to Light-Dark (LD) stimuli. Negative values indicate a predominance of nocturnal activity, while positive results indicate diurnal activity.

Evaluating the responses of rhythms

The data obtained from the Hurst coefficient, analyzed over the three-day period (H) (Fig. 6), for the first day (Hi) (Fig. 7), and for the last day (Hf) (Fig. 8), unveiled a consistent trend in rhythms, suggesting the presence of a pattern. The variability among the assessed values was more noticeable in the LD treatment, with no significant evidence of variation among the species across treatments, as indicated by the Hurst coefficient (Table 3).

Table 3. Variation among treatments in different types of treatments (LL, LD, and DD), Hurst coefficient and (IS) is the intermediate stability calculated for LD, to assess the level of synchronization.

	Treatment	F(value) / Chi-Square(χ^2)	*p* -value
H	DD	F(4,31)= 0.246	0.910
	LL	F(4,40)=0.034	0.988
	LD	F(4,39)=1.423	0.245
Hi	DD	F(4,31)=0.607	0.661
	LL	F(4,40)=0.316	0.865
	LD	χ^2= 12.173	0.016*
Hf	DD	F(4,31)=0.357	0.837
	LL	F(4,40)=0.362	0.834
	LD	F(4,39)=0.813	0.525
IS	LD	F(4,39)=1.454	0.235

** In the LD analysis for the "Hi" approach using the Kruskal-Wallis test, it was observed that the species *I. iuiuensis* in conjunction with *X. santinhoi* (*p* = 0.057) and *I. iuiuensis* in conjunction with *Xangoniscus* sp.2 (*p* = 0.019) exhibited differences in temporal patterns.

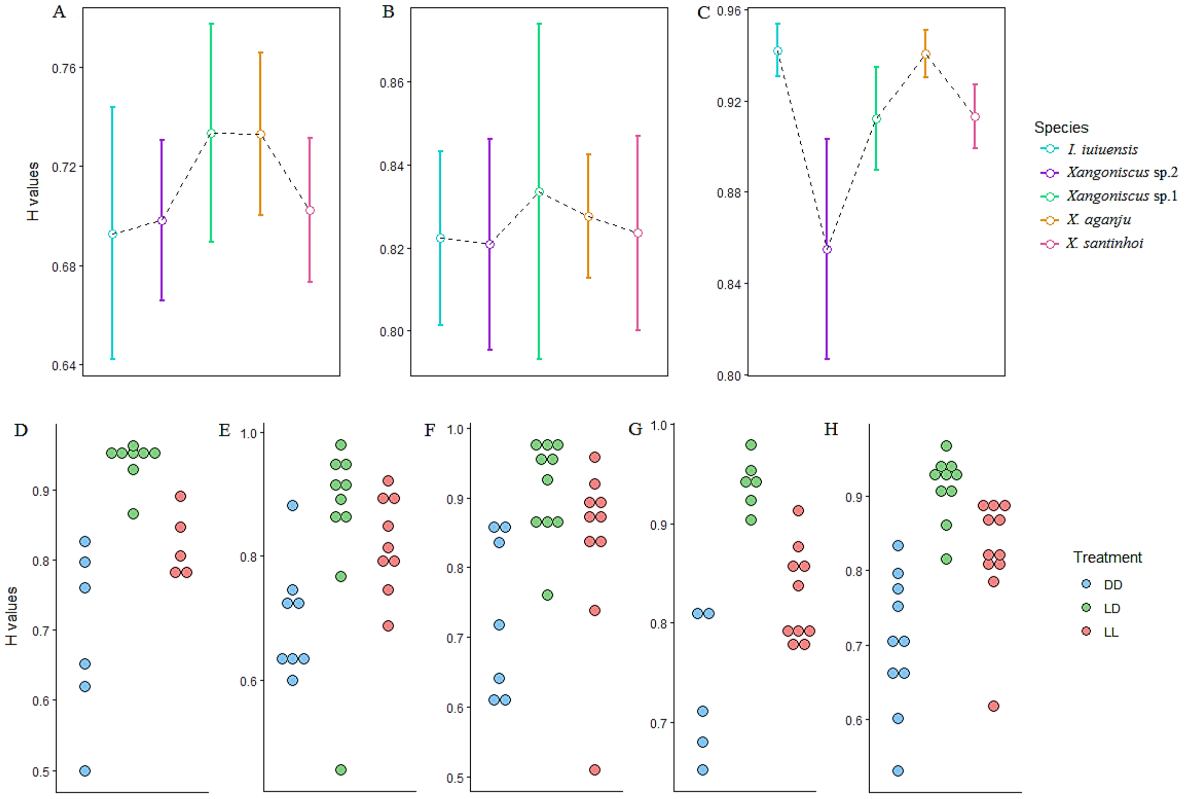

Fig. 6. Variation in data for each species over three days, applied to the Hurst coefficient (H), with a comparison between treatments (A) DD, (B) LL, and (C) LD. The variability among different light regimes is also illustrated for each species: (D) *I. iuiuensis*, (E) *Xangoniscus* sp. 2, (F) *Xangoniscus* sp.1, (G) *X. aganju*, and (H) *X. santinhoi*.

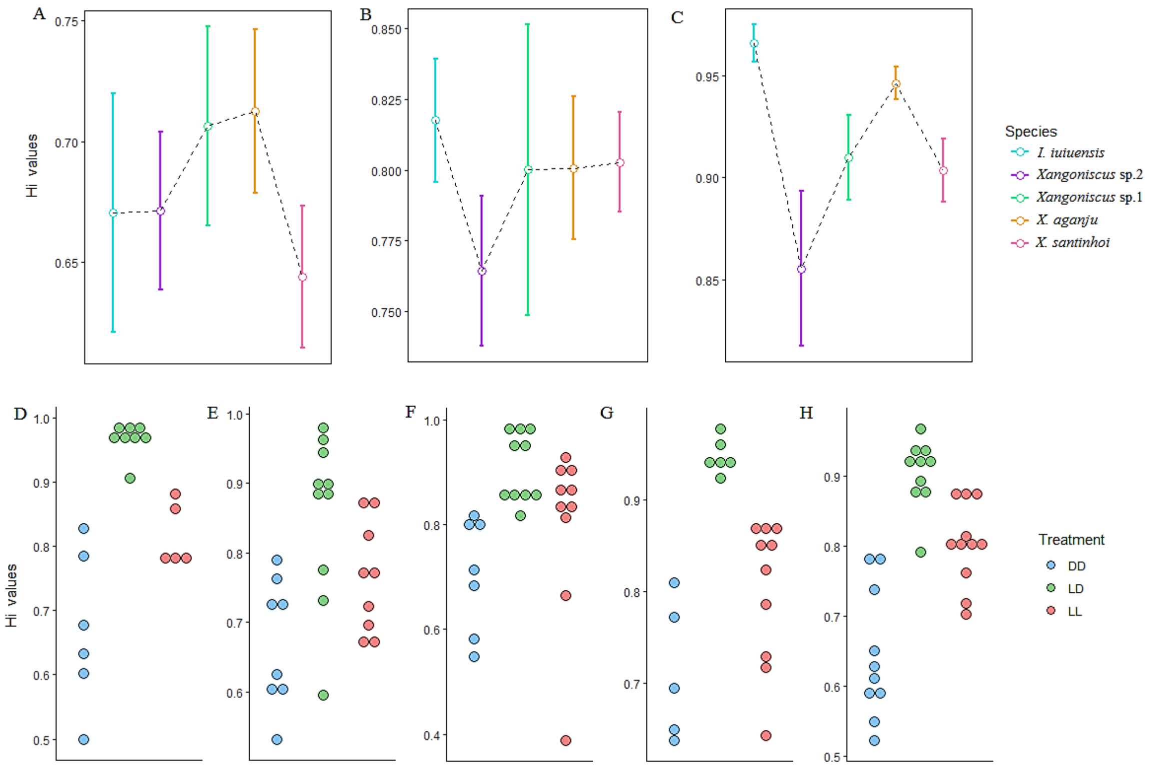

Fig. 7. Variation in data for each species on the first sampled day, applied to the Hurst coefficient (Hi), with a comparison between treatments (A) DD, (B) LL, and (C) LD. The variability among different light regimes is also illustrated for each species: (D) *I. iuiuensis*, (E) *Xangoniscus* sp. 2, (F) *Xangoniscus* sp.1, (G) *X. aganju* e (H) *X. santinhoi*.

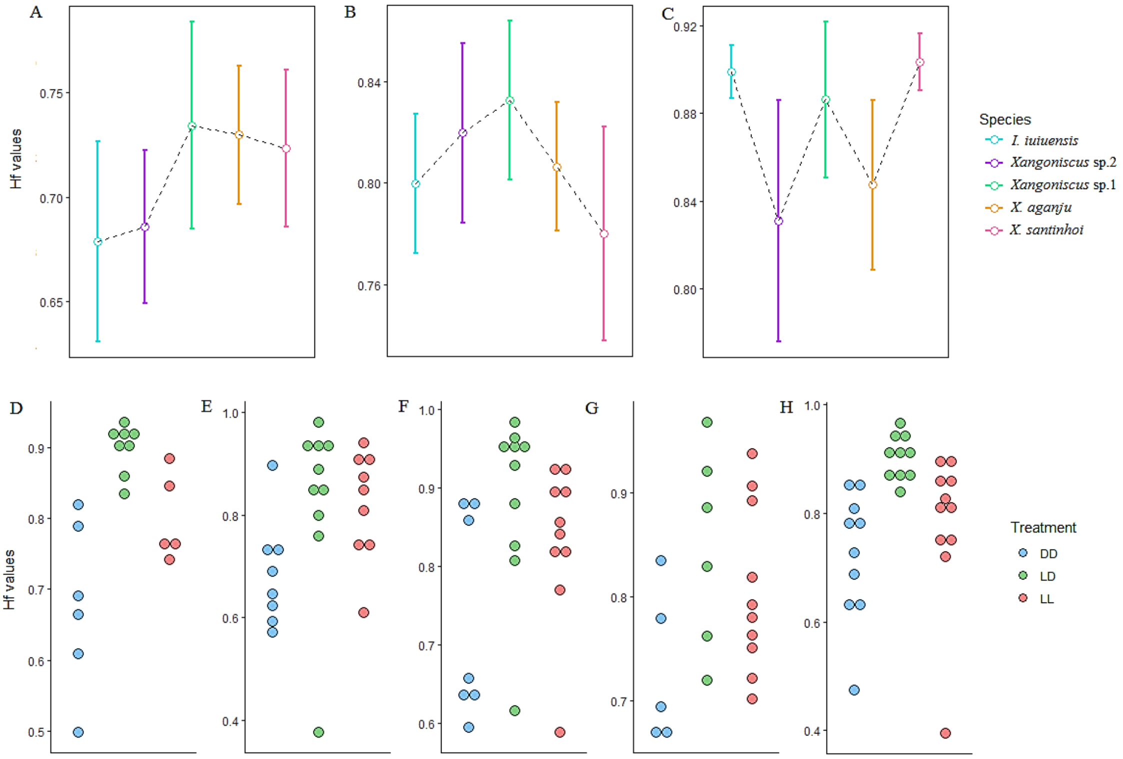

Fig. 8. Variation in data for each species on the third day of recording, applied to the Hurst coefficient (Hf), with a comparison between treatments (A) DD, (B) LL, and (C) LD. The variability among different light regimes is also illustrated for each species: (D) *I. iuiuensis*, (E) *Xangoniscus* sp. 2, (F) *Xangoniscus* sp.1, (G) *X. aganju* e (H) *X. santinhoi*.

However, upon evaluating the adaptability of rhythm under the DD, LL, and LD regimes among individuals of the same species (D, E, F, G, and H in Figs. 6-8), notable discrepancies in value distributions ($p < 0.05$) were obtained for all species except for *Xangoniscus* sp. 2 and *X. aganju* regarding Hf (Table 4). Table 5 highlights the relationship of treatments within each species.

Table 4. Variation in values of H, Hi, Hf, and IS among species subjected to DD, LL, and LD.

	Species	**F(value) / Chi-Square(χ^2)**	***p*-value**
IS	*Iuiuniscus iuiuensis*	F(2,16)= 22.16	2.45e-05
	Xangoniscus sp. 1	F(2,24)= 32.06	1.67e-07
	Xangoniscus santinhoi	F(2,28)= 30.19	1.03e-07
	Xangoniscus sp. 2	F(2,24)= 20.62	6.14e-06
	Xangoniscus aganju	F(2,18)= 7.66	3.92e-03
H	*Iuiuniscus iuiuensis*	χ^2= 13.894	9.61e-04
	Xangoniscus sp. 1	F(2,24)= 5.77	9.02e-03
	Xangoniscus santinhoi	F(2,28)= 20.17	3.75e-06
	Xangoniscus sp. 2	F(2,28)= 4.45	0.023
	Xangoniscus aganju	χ^2= 13.57	1.13e-03
Hi	*Iuiuniscus iuiuensis*	χ^2= 14.60	6.75e-04
	Xangoniscus sp. 1	F(2,24)=6.04	7.48e-03
	Xangoniscus santinhoi	F(2,28)=36.07	1.79e-08
	Xangoniscus sp. 2	F(2,24)=7.61	2.76e-03
	Xangoniscus aganju	F(2,18)=14.56	1.73e-04
Hf	*Iuiuniscus iuiuensis*	12.77	1.69e-03
	Xangoniscus sp. 1	F(2,24)=3.75	0.038
	Xangoniscus santinhoi	F(2,28)=6.96	3.51e-03
	Xangoniscus sp. 2	F(2,24)=3.03	0.067
	Xangoniscus aganju	F(2,18)=2.77	0.089

Table 5. The study presents differences in values for the H, Hi, Hf, and IS tests among the DD, LL, and LD treatments for each species., interpreted as distinct when *p* < 0.05.

	Species	Treatment	*p* -value		Species	Treatment	*p* -value
IS	*I. iuiuensis*	LD-DD	6.43e-05	**Hi**	*I. iuiuensis*	LD-DD	5.76e-04
		LL-DD	0.941			LL-DD	0.710
		LL-LD	2.32e-04			LL-LD	0.069
	Xangoniscus sp. 1	LD-DD	6.63e-06		*Xangoniscus sp. 1*	LD-DD	5.91e-03
		LL-DD	0.869			LL-DD	0.272
		LL-LD	4.01e-06			LL-LD	0.124
	X. santinhoi	LD-DD	4.40e-07		*X. santinhoi*	LD-DD	1.10e-08
		LL-DD	0.840			LL-DD	3.82e-05
		LL-LD	1.25e-06			LL-LD	6.48e-03
	Xangoniscus sp. 2	LD-DD	4.71e-05		*Xangoniscus sp. 2*	LD-DD	1.92e-03
		LL-DD	0.999			LL-DD	0.155
		LL-LD	2.70e-05			LL-LD	0.136
	X. aganju	LD-DD	0.003		*X. aganju*	LD-DD	1.39e-4
		LL-DD	0.197			LL-DD	0.071
		LL-LD	0.044			LL-LD	4.49e-03
H	*I. iuiuensis*	LD-DD	4.20e-04	**Hf**	*I. iuiuensis*	LD-DD	1.42e-03
		LL-DD	0.385			LL-DD	0.757
		LL-LD	0.038			LL-LD	0.108
	Xangoniscus sp. 1	LD-DD	6.52e-03		*Xangoniscus sp. 1*	LD-DD	0.030
		LL-DD	0.161			LL-DD	0.202
		LL-LD	0.246			LL-LD	0.546
	X. santinhoi	LD-DD	2.25e-06		*X. santinhoi*	LD-DD	0.003
		LL-DD	2.41e-03			LL-DD	0.477
		LL-LD	0.027			LL-LD	0.042
	Xangoniscus sp. 2	LD-DD	0.021		*Xangoniscus sp. 2*	LD-DD	0.080
		LL-DD	0.091			LL-DD	0.124
		LL-LD	0.796			LL-LD	0.982
	X. aganju	LD-DD	5.81e-04		*X. aganju*	LD-DD	0.077
		LL-DD	0.264			LL-DD	0.239
		LL-LD	9.63e-03			LL-LD	0.617

In IS, higher values were recorded in LD, indicating a more synchronized rhythm compared to other conditions for all species (*I. iuiuensis* > *Xangoniscus* sp.1 > *Xangoniscus* sp.2 > *X. santinhoi* > *X. aganju*). There was no significant evidence of variability among species regarding the light/dark treatment (Table 3).

However, when assessing the versatility of the rhythm under the DD, LL, and LD regimes among individuals of the same species (Fig. 9B-F), significant differences in value distributions were observed (*p* < 0.05), except for LL-DD in all species (Table 4), due to the irregularity of the rhythm.

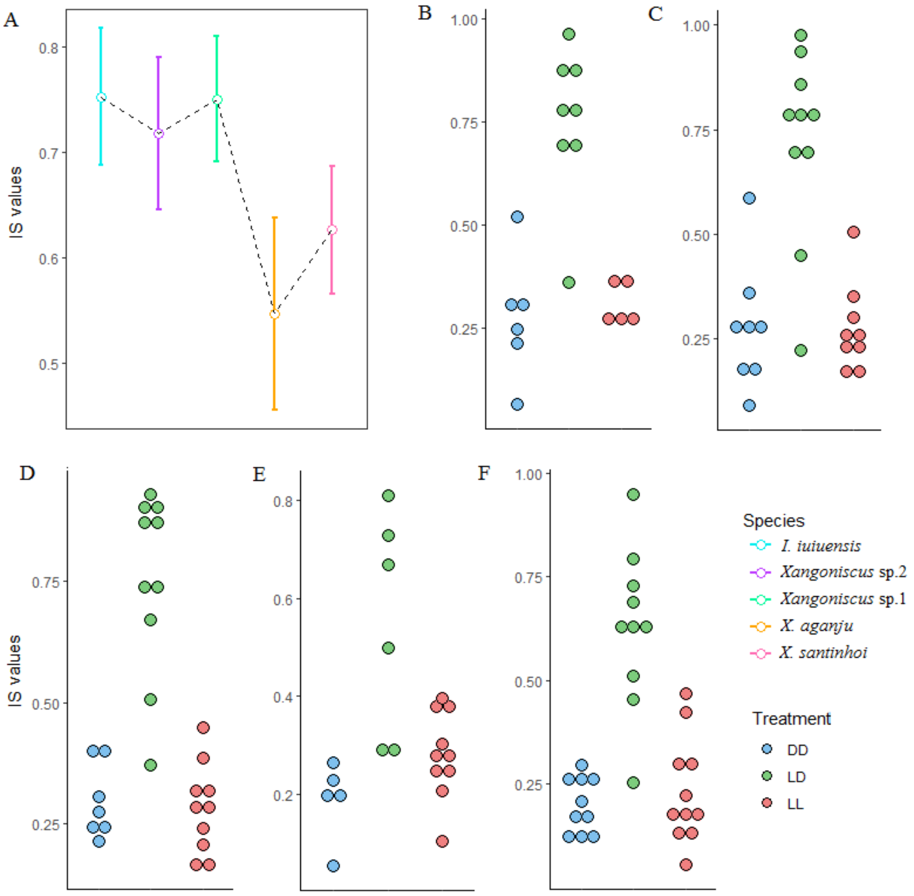

Fig. 9. Variation in the IS values for each isopod species under LD conditions. The variability among different light regimes is also illustrated for each species: (B) *I. iuiuensis*, (C) *Xangoniscus* sp. 2, (D) *Xangoniscus* sp.1, (E) *X. aganju* e (F) *X. santinhoi*.

DISCUSSION

Contradicting the hypothesis, all five isopod species exhibited rhythmic patterns closely resembling circadian rhythms under light-dark (LD) conditions, suggesting no loss of rhythmic elements, indicating that they did not become aperiodic in their locomotor activity. However, under constant light (LL) and constant darkness (DD) regimes, the species appeared asynchronous, displaying more infradian rhythms with distinct amplitudes, albeit without clearly defined rest periods.

This pattern differs from the study by Oda *et al*. (2000), for instance, where infradian rhythms in DD, related to cuticular molting and oviposition in cave-dwelling springtails, remained constant across coupling phases, possibly attributable to a long period of isolation (Trajano e*t al*., 2009).

According to Strauss and Dircksen (2010), there is evidence suggesting that certain circadian rhythms, particularly in specific developmental stages of crustaceans, stem from the coupling of higher frequency ultradian oscillators. The proposed hypothesis posits that circadian rhythms are predominantly derived from ultradian rhythms, potentially elucidating the presence of amplitudes with ultradian characteristics as observed in some individuals in this study.

Research on terrestrial and marine isopods has revealed the presence of circadian rhythms in their behavior, with a peak in locomotor activity at night (Smith and Larimer, 1979). These rhythms are driven endogenously and persist under constant conditions, such as tidal frequency (Jones and Naylor, 1970), differing from the isopods in the current study, where circadian rhythm under constant conditions was not as pronounced.

Light pulses demonstrate the ability to synchronize circadian rhythms when administered to organisms in a persistently dark environment, especially at intervals corresponding to the natural transitions of the traditional light-dark photoperiod (LD), as observed in the studied isopods. This influence can extend to locomotor and reproductive behaviors, harmoniously coordinating the functioning of sensory organs, nervous structures, metabolism, and developmental processes (Oda *et al*., 2000; Strauss and Dircksen, 2010).

In other crustaceans, such as the decapod *Orconectes pellucidus*, a diversity of behaviors has been revealed in previous studies. In the work of Park *et al*. (1941), arrhythmia was identified in DD, LD, and LL, but subsequent research uncovered significant variations beyond negative phototaxis. For instance, in the study by Brown (1961), the presence of both circadian and infradian rhythms (only in DD) was observed. Conversely, studies on respiratory activity and reproductive cycles (Jegla and Poulson, 1968; 1970) highlighted a circadian rhythm. On the other hand, the troglobitic decapod *Procambarus cavernicola* exhibited a locomotor activity pattern closer to circadian in all three types of regimes (O-Martínez *et al*., 2004).

The amphipods of the genus *Niphargus*, both the non-troglobitic *N. puteanus* and the troglobitic *N. poianoi*, exhibited similar responses in locomotor activity, showing both ultradian and circadian rhythms under constant darkness (DD) conditions (Blume *et al*., 1962; Auletta *et al*., 2017). Additionally, under light-dark (LD) conditions, synchronization with the circadian rhythm was observed, aligning with the behavior of the five species in the present study.

Habitat selection involves a range of behaviors directing individuals to occupy specific areas, aiming to maximize their fitness (Holt and Barfield, 2008). Semi-aquatic or subterranean amphibious species show strong spatial segregation, even in the absence of physical barriers, and may distribute randomly, driven by directional adaptive divergence (Fišer *et al*., 2016; Worsham *et al*., 2023). This isolation allows these species to manifest distinct behaviors, even within similar groups. This variation can be explained by differences in activity periods, with species further south, in the state of Minas Gerais (*Xangoniscus santinhoi* and *Xangoniscus* sp. 1) exhibiting nocturnal activity, while those located further north, in the state of Bahia, displayed diurnal behavior.

Reproductive behaviors, specific feeding habits, and predator evasion strategies shared among organisms cohabiting the same habitat, such as fish, are factors that could potentially contribute to understanding the observed differences in activities, particularly among species of the same genus (McPeek, 1990; Peterson *et al*., 2021). It is hypothesized that the isopods found deep within the caves collected in Bahia may exhibit diurnal behavior, as they are located far from the entrance, thereby placing them out of reach of potential predators from external habitats during the process of evolutionary isolation. In the case of *Xangoniscus* sp. 1, also discovered in a zone distant from the entrance, fluctuations or a reduction in the water level in the cave (Lapa da Lagoinha III) may be indicated. Conversely, *X. santinhoi*, whose individuals can be found even near the cave's entrances, exhibits nocturnal activity, which may serve to evade potential diurnal predators.Some studies have observed a decrease in the behavioral response to light, resembling the regressive evolution of an eye or pigment (Peirson *et al*., 2009). However, experimental evidence for this investigation is varied and inconclusive (O-Martínez *et al*., 2004; Friedrich *et al*., 2011; Friedrich, 2013; Pasquali and Sbordoni, 2014; Worsham *et al*., 2023), because there could potentially exist a non-visual and extraocular detection mechanism similar to that observed in myriapods, where the thermal receptor BRTNaC1 enables the detection of sunlight (Yao *et al*., 2023). In the context of this study, the species appeared to respond to light even with absent eyes, and being found in completely aphotic zones, exhibiting distinct behaviors during exposure to the light-dark cycle. These results emphasize the influence of light on the circadian rhythm of these species, indicating that morphological characteristics adapted to subterranean environments may not be accurate indicators of reduced rhythmicity in troglobites, as suggested by Menna-Barreto and Trajano (2015). Biological clock genes may be conserved in cave species, coordinating rhythmic patterns in response to non-visual cyclic variables (Friedrich, 2013).

According to Worsham *et al*. (2023), the expression of phenotypic characteristics extends beyond morphology, emerging from complex evolutionary processes that enable adaptation to stable environments without necessitating obvious changes in morphology. Shared phenotypes among the studied species, such as the absence of ommatidia and pigmentation, are also evident across a range of organisms, irrespective of their degree of relatedness (Campos-Filho *et al*., 2014; Worsham *et al*., 2023). Therefore, the plasticity in the expression of rhythmicity becomes apparent.

The maintenance of the biological clock in the studied cave-dwelling species can also be explained by the need to coordinate metabolic processes, i.e., the pursuit of homeostasis (Oda *et al*., 2000; Friedrich, 2013). The regression of the visual system in troglomorphs does not automatically imply a regression of the biological clock, as observed. Moreover, it is plausible that the biological clock remains intact in cave species, playing a crucial role in regulating rest patterns (Allada and Siegel, 2008).

The results revealed a pattern in the rhythm, with greater variability in the LD treatment, which showed a rhythm closer to circadian and significant differences in the distribution of values ($p < 0.05$) among species in the treatments (LL, DD, and LD). These findings align with previous studies that also found variability in the expression of circadian rhythms among studied organisms, attributing such differences to adaptation to environmental changes (Rossano *et al*., 2008) and the ability to time-keep in response to light and darkness (Koilraj *et al*., 2000).

In the analysis of Intermediate Stability (IS), values were higher under LD, indicating synchronization of the rhythm across all species. However, when examining rhythmic characteristics among individuals of the same species under all regimes, notable differences in distributions are observed, except for LL-DD due to the irregularity of the rhythm. Studies that also investigated the presence of circadian rhythms in cave-dwelling species, such as the millipede *Glyphiulus cavernicolus* (troglobite) (Koilraj *et al*., 2000) and the amphipod *Orchestia gammarellus* (non-troglobite) (Jelassi *et al*., 2013), revealed significant individual variations in different treatments, with greater stability and activity observed in the LD condition. This suggests that, although the light-dark treatment may not vary significantly among cave species, individual differences in response to different light regimes can result in irregularities in the rhythm.

CONCLUSION

The cave-dwelling species of Styloniscidae subjected to LL, DD, and LD treatments in this study proved to be important for the preliminary understanding of how biological clock functions may operate in cave isopods. Contrary to the initial hypothesis, all species displayed synchronized rhythms (LD) that fluctuated akin to an endogenous rhythm, indicating their capability for light detection and synchronization with this particular stimulus. However, when exposed to constant light (LL) and constant darkness (DD), the species adopted more infradian rhythms, suggesting plasticity. The variation in the concentration of nocturnal and diurnal activity among species emphasizes the diversity in behavioral responses to still misunderstood environmental and ecological stimuli.

The analysis of the spontaneous circadian activity of these troglobiotic organisms provides essential insights into the adaptive significance of possessing a functional circadian clock in unique environments such as caves. Investigating how these circadian rhythms confer advantages in seemingly non-cyclical environments is crucial for a deeper understanding of the behavioral ecology of these species, underscoring the importance of future research aimed at unraveling the underlying mechanisms of the observed rhythms.

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CONFLICT OF INTEREST STATEMENT

The authors have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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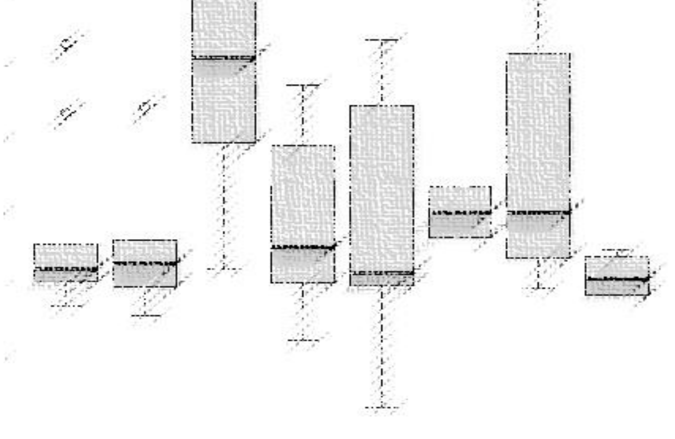
ARTIGO III

WE PREFER NIGHTTIME, THEY PREFER DAYTIME: BIOLOGICAL RHYTHM VARIABILITY IN CAVE-DWELLING AMBLYPYGIDS (AMBLYPYGI: CHARINIDAE) IN THE NEOTROPICS



ARTIGO III

Este manuscrito foi elaborado em sua versão preliminar, seguindo as diretrizes estabelecidas pela revista *Journal of Zoology*

We Prefer Nighttime, They Prefer Daytime: biological Rhythm Variability in Cave-Dwelling Amblypygids (Amblypygi: Charinidae) in the Neotropics

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Abstract

The lack of understanding regarding how endogenous and behavioral factors affect the biological rhythms of amblypygid arachnids in cave environments underscores a gap in chronobiological knowledge for this group. This study investigates the influence of specialization on subterranean habitats and the presence of biological rhythms on the locomotor activity patterns of the troglobitic and troglophilic species of the genus *Charinus*. Specimens collected from carbonate caves in Brazil were subjected to experimental treatments of constant light (LL), constant darkness (DD), and light-dark cycles (LD). The results revealed variations in the distribution of main periods among species, without a uniform pattern. Although some specimens showed greater variability in activity patterns in the DD and LL treatments, no significant differences were observed between troglobites and troglophiles. The lack of a clear distinction in rhythms between the two groups suggests a dynamic nature of circadian rhythms in these populations, with individual variations in activity patterns, indicating behavioral diversity. Additionally, intraspecific competition for food resources, probably intensified by the oligotrophic conditions of the cave environments, may play an important role in shaping these patterns and differences in activity phases. The presence of infradian rhythms and weak circadian rhythms in some individuals underscores the importance of considering non-photic zeitgebers for a deeper understanding of these rhythms in cave organisms.

Keywords: locomotor activity; cave; *Charinus*; Neotropics; arrhythmia; Amblypygi.

Introduction

The order Amblypygi Thorell, 1883 comprises arachnids that inhabit specific microhabitats, including crevices, debris accumulations, bromeliads, the undersides of rocks and tree trunks, as well as caves (Weygoldt 2000; Harvey 2003; Chapin & Hebets 2016; Miranda *et al.*, 2018). Many species demonstrate fidelity to these locations for prey capture, territorial defense, and reproduction. Recognized as predators, these animals have a varied and opportunistic diet, and they may also exhibit necrophagy behavior (Weygoldt 2000; Harvey, 2003; Chapin & Hebets, 2016; Santer & Hebets, 2011; Rivera *et al.*, 2009; Prous *et al.*, 2017; Trujillo *et al.*, 2021).

Chapin and Hebets (2016) observed that amblypygids are more active at twilight, engaging in exploration and foraging while becoming more immobile during the day. In caves, their populations exhibit distinct behaviors in surveillance, habitat preference, and predation compared to epigean species. Acts of predation and surveillance are more pronounced in hypogean animals (Chapin, 2015), and aggression in agonistic behavior among males is of lower intensity. These differences are believed to result from behavioral plasticity in response to the environment in which they live (Miranda *et al.*, 2016; Segovia, Neco & Willemart, 2018).

In recent years, several studies have delved into behavioral aspects of this group, with the majority focusing on species inhabiting epigean environments. These investigations have explored sociability-related issues, including parental care (Rayor & Taylor, 2006; Chapin & Hebets, 2016), intraspecific behavioral divergence concerning agonistic interactions and the environment (Chapin, 2015; Chapin & Hill-Lindsay, 2016), and individual-level recognition (Walsh & Rayor, 2008). Additionally, research in physiology, sensory biology, tactile learning, and the neuroanatomy of amblypygids has addressed gaps related to distal mechanisms (adaptive mechanisms, such as the structure of the sensory anteniform leg being modified over evolution) and proximal mechanisms (physiological and genetic mechanisms) that determine behavior, thus enhancing our understanding of their specialized sensory systems (Tinbergen, 1963; Santer & Hebets, 2009; Santer & Hebets, 2011).

Nevertheless, to date, no studies have offered a comprehensive understanding of how endogenous and behavioral factors influence the biological rhythms of these animals in cave environments. This gap poses significant limitations in understanding their activity patterns, emphasizing the urgent need for further research to gain deeper insights into the functioning of the biological clock in natural subterranean ecosystems.

Caves represent ecosystems with unique conditions compared to the surface, characterized by climatic constancy, a tendency towards oligotrophy, and the absence of light-dark cycles. These environments have distinct zones marked by the presence or absence of light along their length (Poulson & White, 1969; Howarth, 1983; Moldovan, Kovac & Halse, 2018; Culver & Pipan, 2019). Cave temperatures resemble the external annual average, while air humidity exceeds surface levels, potentially reaching saturation (Hüppop, 2000). These patterns underscore the singularity and dynamics of these subterranean environments, serving as biological filters that select species with varying degrees of adaptation and persistence in such conditions (Poulson & White, 1969; Culver & Pipan, 2009; Mammola, 2019).

Non-obligate cave species maintain their visual systems, including sensory organs and parts of the central nervous system. Their daily activities are regulated by circadian stimuli, and variations in these activities may indicate the influence of environmental factors on the synchronization of biological rhythms, adjusted through phenotypic convergences (Oda *et al.*, 2000; Duboué *et al.*, 2012; Friedrich, 2013). Conversely, obligate cave fauna, or troglobites, are highly specialized, often exhibiting a complete absence of vision and pigmentation, along with elongated appendages, specialized metabolism, and enhanced sensory systems (Trajano *et al.*, 2009; Pasquali & Sbordoni, 2014; Beale, Whitmore & Moran, 2016). These shared characteristics have undergone extensive evolutionary processes following the colonization of epigean populations into the cave environment (Pasquali & Sbordoni, 2014).

Caves are often regarded as potentially arrhythmic environments, representing some of the most stable habitats on the planet. However, the persistence of rhythmicity in organisms inhabiting such environments raises questions about its purpose and significance, particularly considering its independence from any coordination of the animal's physiology with external environmental cues (Beale *et al.*, 2016). Despite this, there is a paucity of studies dedicated to investigating the rhythms of cave-dwelling organisms (*e.g.*, Hoenen & Marques, 1998; Hoenen & Gnaspini, 1999, Oda *et al.*, 2000; Gnaspini, Santos & Hoenen, 2003; Trajano *et al.*, 2009; Friedrich, 2013; Abhilash, Shindey & Sharma, 2017; Royzenblat, Kulacic & Friedrich, 2023), particularly those that consider differences in experimental conditions (Beale *et al.*, 2016) and those that compare troglobiotic and non-troglobiotic invertebrate species that are closely related (Friedrich, 2013; Pasquali & Sbordoni, 2014).

Given the provided information, our study aimed to investigate whether the degree of specialization and the presence of rhythmic elements influence the locomotor activity patterns of troglobitic and troglophilic (non-obligate, capable of establishing populations on the surface) amblypygid species belonging to the genus *Charinus*. This genus boasts a wide distribution, currently comprising 97 species (Miranda *et al.*, 2021). In Brazil, nearly 45% of these species have been recorded (Souza *et al.*, 2024), with 22 of them identified in cave environments (Weygoldt, 2000; Chapin & Hebets, 2016; Miranda *et al.*, 2018; Miranda *et al.*, 2021). Due to their small size and abundance, this group emerges as a promising model for studies in ecological, evolutionary, and behavioral fields. Additional advantages include ease of collection and ex-situ breeding (Hoenen & Gnaspini, 1999; Foelix & Hebets, 2001; Vasconcelos, Giupponi & Ferreira, 2014; Vasconcelos *et al.*, 2016; Moldovan *et al.*, 2018; Segovia, Gainett & Willemart, 2020; Miranda *et al.*, 2021).

Based on this, we formulated the following hypotheses: *i)* it is expected that troglobitic species exhibit a weak or absent circadian rhythm, thus demonstrating asynchrony in their activity patterns; *ii)* troglophilic species are expected to show a more pronounced circadian rhythm and tend to synchronize their activity patterns with the light-dark cycle.

Materials and methods

Sampled Species and Collection sites

The study included four troglophilic species and four troglobitic species of the genus *Charinus*. The troglophiles were represented by the following species (Fig.1A–D): *Charinus santanensis*, *C. jibaossu*, *C. potiguar*, and *C. iuiu*. Whereas the species *Charinus eleonorae*, *C. spelaeus*, *C. taboa*, and *C. troglobius* are troglobites (Fig.1E–H).

It is noteworthy that the troglobitic species used in this study exhibit distinct traits associated with their subterranean lifestyle, known as troglomorphisms. These traits include reduced pigmentation, diminishment of median eyes, underdeveloped or reduced lateral eyes, and pedipalps adorned with elongated spines. However, the intensity of these characteristics may vary among species. For instance, in *C. troglobius*, there is a more pronounced reduction in lateral eyes, along with a strong reduction in median eyes and ocular tubercle (Baptista & Giupponi, 2002). Another shared feature among these species is the arching or inclination of the pedipalp tibia (Haug & Haug, 2021; Schmidt, Melzer & Bicknell, 2022). Conversely, non-troglobites like *C. iuiu*, *C. santanensis*, and *C. jibaossu* possess well-developed lateral eyes and more prominent pigmentation. It is worth noting that *C. potiguar* slightly deviates from this pattern, displaying weaker eye development and ocular tubercle, alongside a slight inclination of the pedipalps (Vasconcelos, Giupponi & Ferreira, 2013). Other traits, such as the non-circular shape of the frontal process and the number of cheliceral denticles, are not necessarily indicative of subterranean habitat restriction. This is evident in species like *C. eleonorae* and *C. jibaossu*, which share similarities in these characteristics despite their distinct adaptations (Vasconcelos, 2014; Vasconcelos & Ferreira, 2016).

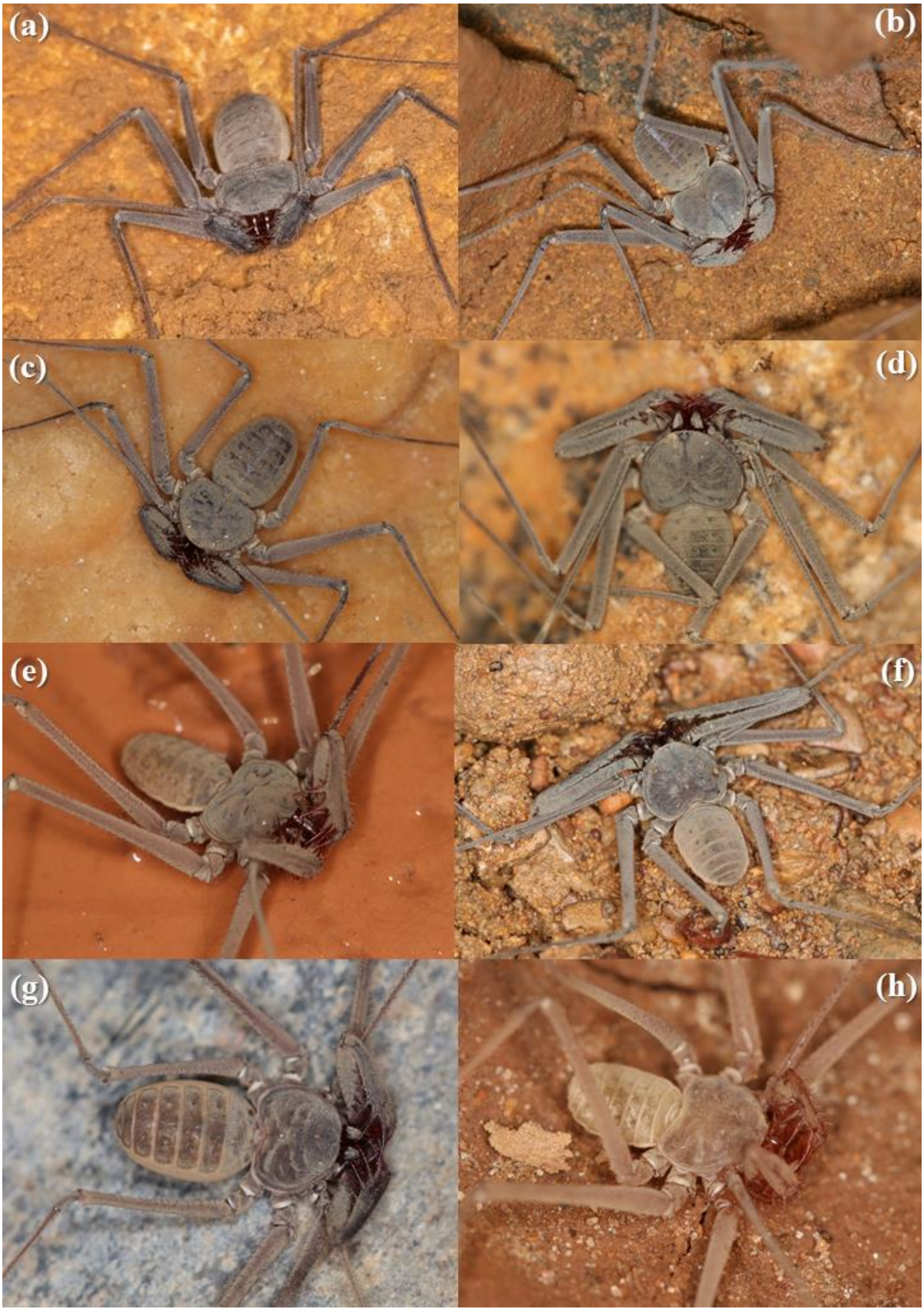

Fig.1. Species chosen and collected for the study: (A) *Charinus potiguar*, (B) *C. iuiu*, (D) *C. santanensis*, (D) *C. jibaossu*, (E) *C. eleonorae*, (F) *C. taboa*, (G) *C. spelaeus*, (F) *C.*

troglobius. Photos by Rodrigo L. Ferreira (B - F) and Diego de M. Bento (A).

All specimens were collected from limestone caves (Fig. 2). *C. potiguar* was collected from Gruta de Martins, also known as Casa de Pedra cave, located in an isolated area of the Seridó geological group, in the municipality of Martins, Rio Grande do Norte. The cave is made of marble and is situated within a geological sub-domain predominantly composed of the Jurucutu formation, characterized by metamorphic rocks (Mascarenhas *et al.*, 2005). The species *C. santanensis* (Gruta do Padre cave), *C. troglobius* (Três Cobras cave), and *C. iuiu* (Toca Fria cave) were collected from caves in the state of Bahia, in the municipalities of Santana, Serra do Ramalho, and Iuiú, respectively. The limestone formation in this region belongs to the Bambuí geological group, known for harboring the largest number of identified caves in Brazil, exceeding six thousand, and covering an area of over 145,000 km² (Auler *et al.*, 2019; Cecav, 2022). For all the species mentioned above, the caves are situated within the Caatinga formation, which is the only semi-arid biome in Brazil. The Caatinga is known for its xerophytic vegetation and distinct seasons, marked by prolonged periods of intense dryness. Also within the Bambuí geological group, the species *C. eleonorae* (Olhos D'Água cave), *C. spelaeus* (Lapa D'Água cave), *C. taboa* (Taboa cave), and *C. jibaossu* (Gruta da Cazanga cave) were collected from caves in the respective municipalities of Minas Gerais: Itacarambi, Presidente Juscelino, Sete Lagoas, and Arcos. These latter species, in contrast to the former ones, were collected from caves located within the Cerrado Biome (Brazilian Savanna), which, although also characterized by well-defined seasons, does not experience the water scarcity observed in the Caatinga.

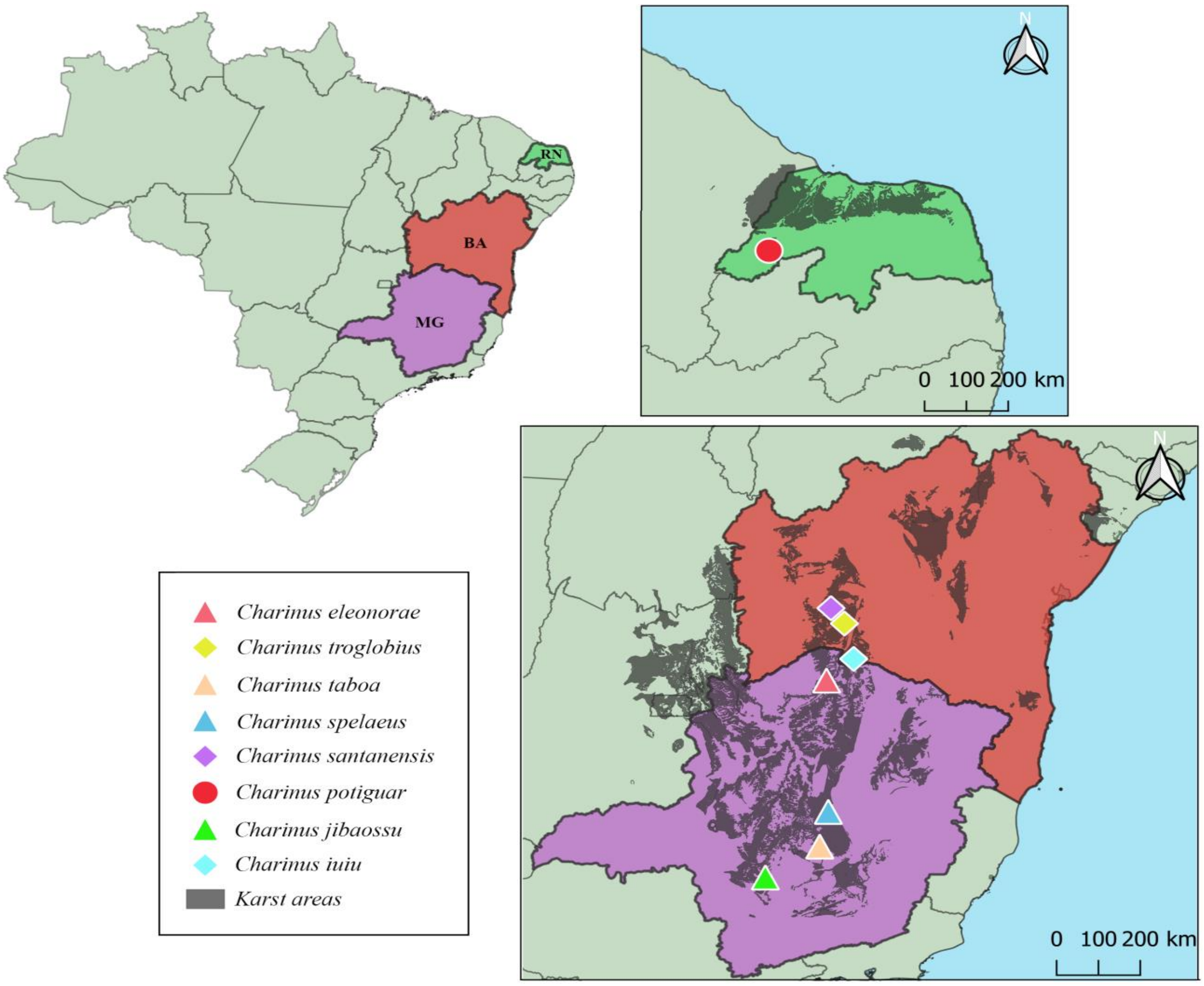

Fig.2. Location of the collection areas of the species chosen and collected for the study, in the municipalities of Martins (RN), Santana (BA), Serra do Ramalho (BA), Iuiu (BA), Itacarambi (MG), Presidente Juscelino (MG), Sete Lagoas (MG), Arcos (MG). In Rio Grande do Norte (above on the right), the karst area of the Apodi geological group is observed, and in Bahia and Minas Gerais (below on the right), the extensive area of the Bambuí geological group is observed.

Manual collections were carried out in the mentioned caves between March and September 2023, with a maximum of 10 individuals per species per cave, in adherence to licensing restrictions imposed by Brazilian authorities, preferably targeting adult specimens. In situ, specimens were carefully isolated in plastic containers and housed in thermal boxes. To maintain humidity levels, cotton balls soaked in water were placed in the container lids.

During transportation, individuals were kept separate within the containers and then transferred to styrofoam boxes to prevent desiccation and abrupt temperature fluctuations. Following collection, the specimens were relocated to a climate-controlled facility at the Center for Subterranean Biology Studies, under the auspices of the Department of Ecology and Conservation at the Federal University of Lavras, situated in Lavras, Minas Gerais, Brazil. This facility offers optimal conditions for specimen storage and preservation, ensuring a controlled and light-free environment.

Maintenance and Experimental Design

The specimens were accommodated in plastic containers prepared with substrate sourced from their natural habitats, including gravel and sand, to mimic their preferred sheltering conditions (Oliveira & Ferreira, 2014). All plastic containers were then placed in BOD incubators. *Charinus potiguar* specimens were separated from the others due to the higher average annual temperature in their collection sites, approximately 28°C, compared to the others (ranging between 23°C and 24°C). Temperature and humidity levels were meticulously regulated to replicate those found in the individuals' original habitats, adjusted through settings in the BOD incubators, and continuously monitored using a digital thermohygrometer. As for feeding, each individual received a weekly diet of cockroach nymphs from the genus *Blaberus* sp. or nymphs and crickets from the genus *Endecous* sp. These prey species were also carefully bred and maintained within the laboratory environment.

To analyze the activity and rest patterns of each species, specimens were housed in conical plastic structures lined with a thin layer of moistened sand to maintain humidity. These structures were then covered with glass lids of the same diameter (Fig. 3). The funnels featured small holes for air circulation and water evaporation, while the transparent glass lids were designed to facilitate efficient image capture. For video recording of the animals' movements, a webcam model Aoni ANC HD Lens Camera (8.0 MP) connected to a computer was utilized. The recording was conducted using open-source software ("Arthropod Motion") developed by Voxar at UFPE (Federal University of Pernambuco, Brazil), specifically tailored for this study. This system enabled simultaneous video capture of two species while ensuring individual monitoring of locomotor activity.

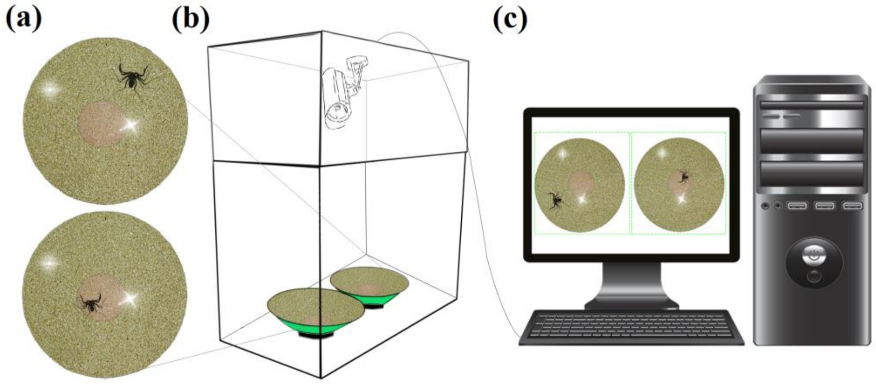

Fig.3. Sampling data structure. (A) Funnels where specimens are housed for recording locomotor rhythms, (B) an aquarium where funnels are kept for filming movements through a webcam attached to a Styrofoam structure, (C) a computer that records movements via the webcam through software.

Before conducting the tests, the specimens underwent a pre-exposure period of three days to the treatment corresponding to what would later be evaluated. During this time, each individual underwent three distinct procedures, with the submission to the free-running treatment (DD, DL, or LL) occurring subsequently. In the first procedure, the environment was immersed in complete darkness (DD), with illumination provided by cold red light lamps positioned 10 cm from the aquariums. Prior to this test, specimens were assessed to confirm their inability to detect red lights. The second procedure took place under constant illumination (Light-Light), with a luminous intensity of approximately 15 lux provided by tubular lamps on the ceiling. The third treatment involved alternating between periods of light and darkness (LD), each lasting 12 hours, controlled by a manual analog timer programmed to alternate the white and red lamps in the room every 12-hour interval. This method ensured a carefully controlled environment to evaluate the behaviors of the animals in response to light and darkness cycles. The administration order of each treatment was randomized.

The locomotor activities of the specimens were recorded over a period of 12 months. During each test, data were captured at intervals of one-tenth of a second and subsequently converted into minutes to cover the entire duration of the experiment. Sampling occurred over a total of 96 non-consecutive days, amounting to 2,304 hours. To prevent potential issues stemming from autocorrelation or pseudoreplication in the datasets, each individual was recorded only once. The number of individuals included varied depending on their availability during collections (with a maximum of ten individuals collected per species due to permit restrictions) and after the ex situ acclimatization period (Table 1).

Table 1. Number of individuals per species tested for each treatment.

Species	**Specimen Qty**		
	LL	**DD**	**LD**
Charinus eleonorae	10	10	10
Charinus taboa	6	8	4
Charinus spelaeus	5	4	2
Charinus potiguar	9	10	10
Charinus iuiu	10	10	10
Charinus santanensis	6	9	6
Charinus jibaossu	5	5	5
Charinus troglobius	7	6	9

Data Analysis

Waveform graphs were generated to illustrate activity patterns during specific time intervals, depicting the mesor at the onset of the activity phase (ascending mesor) and preceding the resting period (descending mesor). The average locomotor activity wave was represented on a dual LD scale. Additionally, the Sokolove and Bushell chi-square periodogram test (1978) was employed, involving the smoothing of derived movement values' means. This methodology is crucial for identifying circadian rhythms within temporal datasets, facilitating the differentiation between random and periodic patterns. The software "El Temps©" version 1.276 was utilized to perform these analyses.

The periodograms were converted into density plots to visually underscore the primary activity periods of troglobites and troglophiles. This method aimed to distinguish unique temporal traits between these two groups while consistently highlighting subtleties within their behavioral patterns. The normality of the data distribution was assessed using the Shapiro-Wilk test. Subsequently, a non-parametric Mann-Whitney test was employed to detect differences in means between the two groups (Mann & Whitney, 1947).

The numerical data collected over three days were converted from tenths of a second to minute-by-minute intervals, and locomotor activity analysis was conducted using MATLAB R2021a software. Throughout the three days of recording, during the light-dark (LD) cycle, light and dark periods were segregated, and the overall mean was computed based on the individuals' movements. Additionally, an analysis of variation (ANOVA) in activity concerning the light-dark cycle was performed by determining the average number of movement points during both the dark and light phases, followed by calculating the difference between them. This assessment facilitated the observation of rhythm stability progression, meaning a regularity in activity patterns over the days, and highlighted consistency in individual profiles. (Witting *et al.*, 1990).

Using the same software, an analysis of the robustness of locomotor activity synchronization level concerning the LD condition was conducted, calculating the Intermediate Stability (IS). This evaluation enabled the observation of rhythm stability progression over the days and e

mphasized consistency in individual profiles (Witting *et al.*, 1990). According to Galasso *et al.* (2019), this methodology offers a more comprehensive examination of activity dynamics over a specific period.

Furthermore, the calculation of the Hurst exponent (H) was employed to examine the presence of long-range dependence in the time series. This metric was utilized in the study to explore scale invariance in *Charinus* locomotor activity patterns under various experimental lighting conditions. Three perspectives were adopted in this investigation. The first (H) allowed for the assessment of the time series over the course of three days. The second (Hi) aimed to examine behavior on the initial day, without any adaptation to the environment (Fig. 3A). The final analysis (Hf) was conducted on the last day, specifically to assess the adaptation of individuals from each species. The determination of the Hurst coefficient was based on the utilization of the first value (r1) with the logarithm base 2 of the autocorrelation function (Eke *et al.*, 2002).

An analysis of variance (ANOVA) was conducted to examine the mean performance concerning Hurst and IS calculations. Subsequently, post-ANOVA comparisons were carried out using the Tukey test. If homoscedasticity, confirmed by the Levene test prior to ANOVA, was not achieved, the data underwent transformation using the logarithmic function. In cases where variances remained unequal even after transformation, the non-parametric Kruskal-Wallis test was employed, with an appropriate Bonferroni correction (Wang *et al.*, 2016; Nwobi & Akanno, 2021). A significance level of *p*-value = 0.05 was established for all analyses, including t-tests, ANOVA, and visual representations, all generated through R software version 4.2.3.

Results

Periodicity and variability of biological rhythms among the species.

The distribution of primary periods among individuals of each species did not display a uniform concentration (Fig. 4). In the DD and LL treatments, no clear trend was observed regarding the primary period. However, it is worth noting that both *C. eleonorae* and *C. spelaeus* exhibited higher variability in the data in the presence of light. Conversely, *C. troglobius*, *C. santanensis*, and *C. iuiu* demonstrated a more pronounced concentration around 1500 minutes in this same treatment.

In the LD treatment context, *C. jibaossu* and *C. eleonorae* exhibited a more pronounced concentration around the mean, while *C. spelaeus*, with only two individuals tested, also displayed a similar trend, suggesting similarity in their distributions. Conversely, the other species showed significant dispersion concerning the primary period. However, *C. taboa* did not exhibit a discernible trend in the distribution of data. The remaining species displayed a more pronounced concentration between 1200 minutes and 1500 minutes, indicating a synchronization of the circadian rhythm. Additionally, *C. eleonorae* revealed higher variability in the values of the primary period, as evidenced by secondary curves.

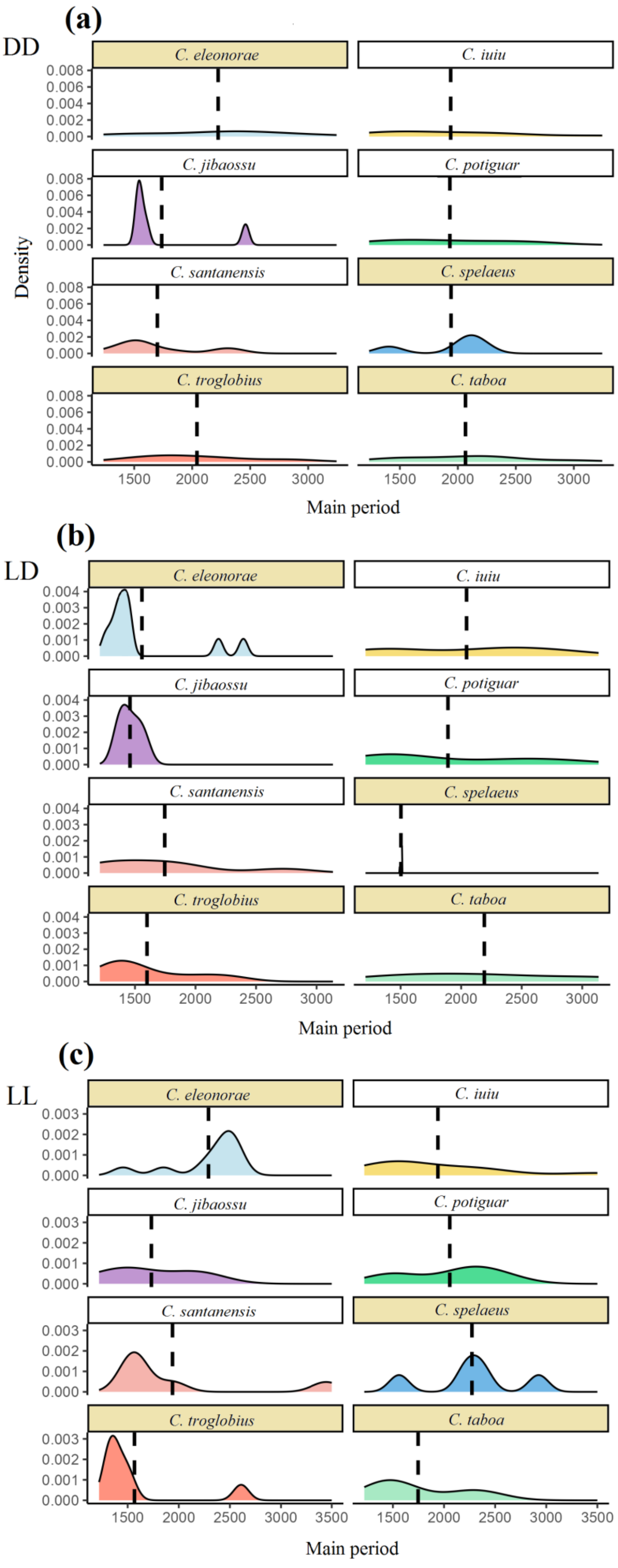

Fig.4. Distribution of main periods among the sampled species, with the troglobitic species *C.*

eleonorae, *C. troglobius*, *C. taboa*, and *C. spelaeus* identified in yellow. A) Density curves in the distribution of periodogram data for the DD treatment, B) LD, and C) LL. The dashed vertical line represents the mean of the raw values.

Throughout the three consecutive days of assessment under the LD treatment, the averages of the waves, determined by the maximum peaks of individuals, did not exhibit a clear pattern in most species (see Appendix I). However, *C. jibaossu* notably did not display a distinct resting period, suggesting a lack of synchronization of the circadian rhythm.

Differences between the main periods of activity in troglobites and troglophiles.

According to the Mann-Whitney analysis, no significant differences were detected in the distribution of main period values between troglobites and troglophiles (see Fig. 5). The findings suggest a lack of substantial evidence for data variation in DD (*p*-value = 0.077; W = 350.5) and minimal evidence in LL (*p*-value = 0.750; W = 399) and LD (*p*-value = 0.266; W = 455).

In the DD treatment, the median for troglophilic species is 1608 (circadian), whereas for troglobitic species, it is 2152 (infradian). In LL, the median for troglophilic species is 1638 (circadian), compared to 2218 (infradian) for troglobitic species. Lastly, in LD, the median for troglophilic species is 1520 (synchronized), while for troglobitic species, it is 1445 (synchronized).

There was no consistent pattern in determining the rhythm type; all species displayed individuals with both infradian and circadian periods in the DD and LL treatments. However, in the LD treatment, only *C. jibaossu* and *C. spelaeus* strictly adhered to a circadian rhythm.

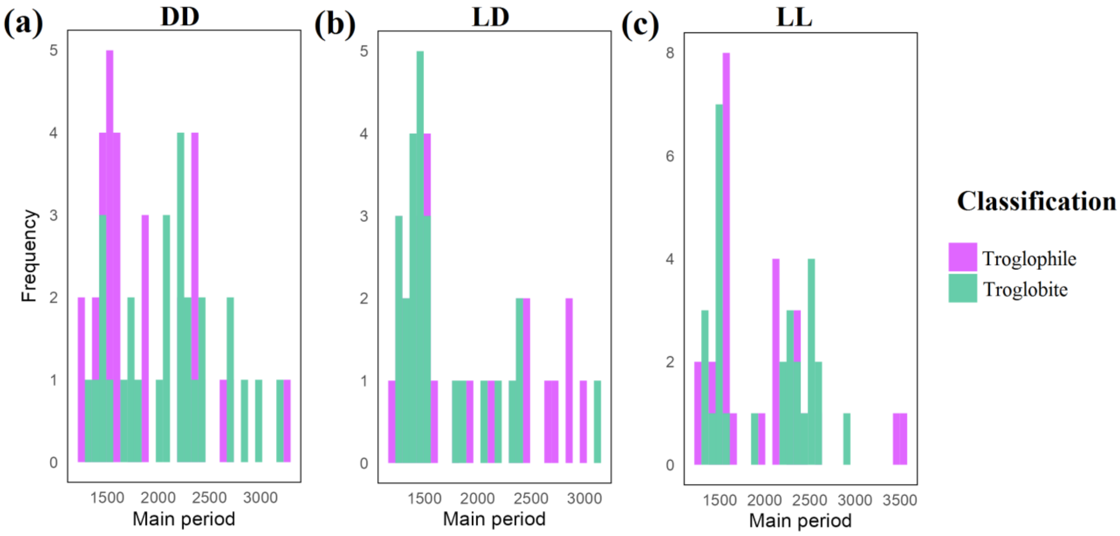

Fig.5. Frequency distribution of periodicity values determined by the main periods between troglophilic and troglobitic species, for the treatments (A) DD, (B) LD, and (C) LL.

Differences in individual activity

Based on the LD treatment data, no distinct patterns or significant variations were observed between the two ecological-evolutionary categories (Fig. 6). However, all individuals of *Charinus santanensis* exhibited diurnal activity, and over 80% of individuals of *C. iuiu* and *C. eleonorae* were also diurnal. Only *C. jibaossu* and *C. troglobius* showed a higher proportion of individuals with nocturnal activity. The remaining species (*C. potiguar*, *C. spaeleus* and *C. taboa*) displayed individuals with both diurnal and nocturnal activities, without a clear preference for a specific activity period.

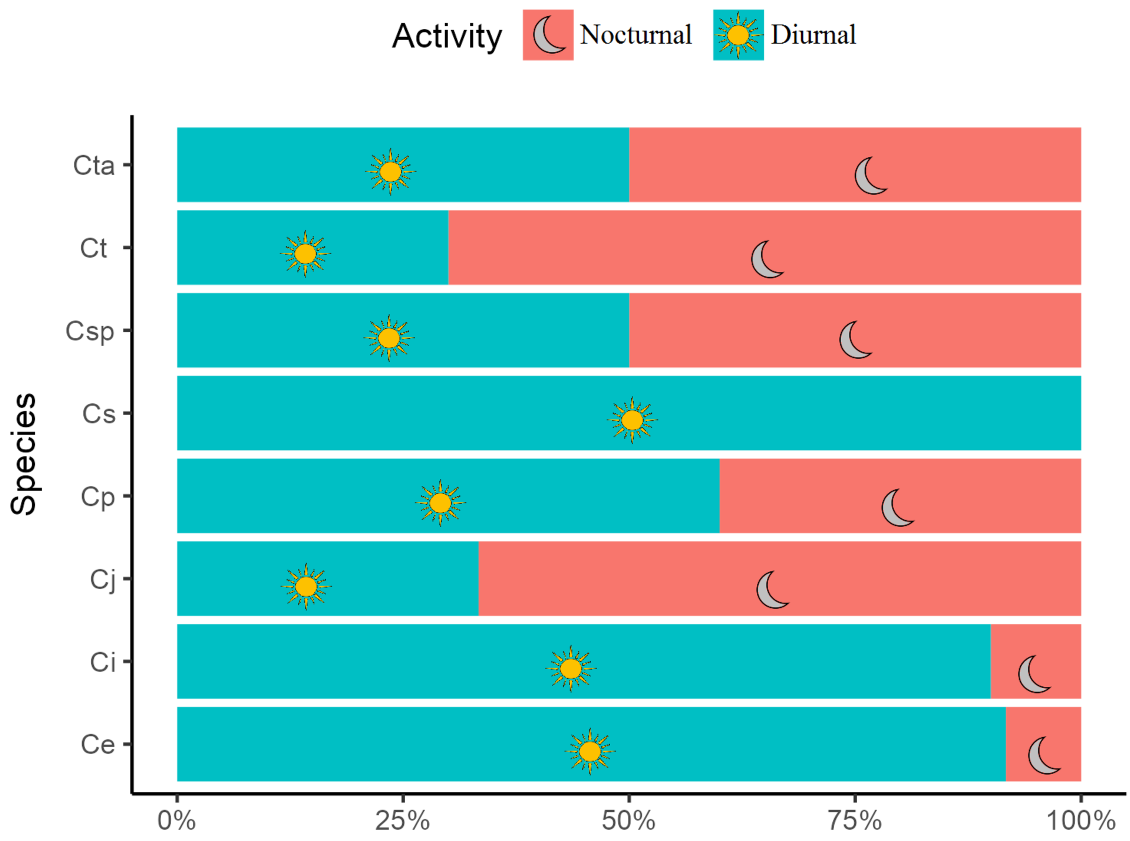

Fig.6. Percentage difference in activity of locomotor activity between individuals of troglobitic and troglophilic species of *Charinus* in response to the light-dark condition (12h Light - 12h Dark).

Cta = *C. taboa*; Ct = *C. troglobius*; Csp = *C. spelaeus*; Cs = *C.santanensis*; Cp = *C. potiguar*; Cj = *C. jibaossu*; Ci = *C. iuiu*; Ce = *C. eleonorae*

Verification of rhythm response

Regarding the degree of rhythm synchronization in LD (see Appendix I), no significant evidence of variability among species was observed [*p*-value = 0.118, F(7,46) = 1.763]. Interestingly, among the species *C. eleonorae*, *C. iuiu*, *C. spelaeus*, and *C. taboa*, only the troglophilic species *C. iuiu* exhibited less variability in values compared to the median.

The data derived from the Hurst coefficient analyzed for the three days (H) (Fig. 7a-c), solely for the first day (Hi) (Fig. 7d-f), and for the last day (Hf) (Fig. 7g-i), generally depicted a consistent rhythmic trend (H > 0.5). Variability among the assessed values was noted in all treatments, with no significant evidence of variation among species regarding treatments for the last day. However, over the three days, only the DD treatment showed significance (Table 2), with differences observed between the species *C. potiguar*, *C. eleonorae*, and *C. iuiu* (Cp-Ce *p*-value = 0.006; Cp-Ci *p*-value = 0.008 and Cs-Cp *p*-value= 0.005). For the first day, only the LD treatment did not demonstrate significance regarding data variability, with differences between the species *C. santanensis* and *C. potiguar* for DD (Cs-Cp *p*-value = 0.013) and for the LL treatment, only *C. jibaossu* and *C. taboa* did not exhibit a difference compared to the others (Ct-Cs *p*-value = 0.032; Cs-Cp *p*-value = 0.002; Csp-Cp *p*-value = 0.009; Cs-Ci *p*-value = 7.4e-4; Csp-Ci *p*-value = 0.005 and Ci-Ce *p*-value = 0.04).

However, when examining the flexibility of rhythm among species and their individuals, it becomes apparent that only during the H stage, particularly in the LL treatment, a clearer trend emerges in its values within the established range, with the data clustering closer to the median (Fig. 7c).

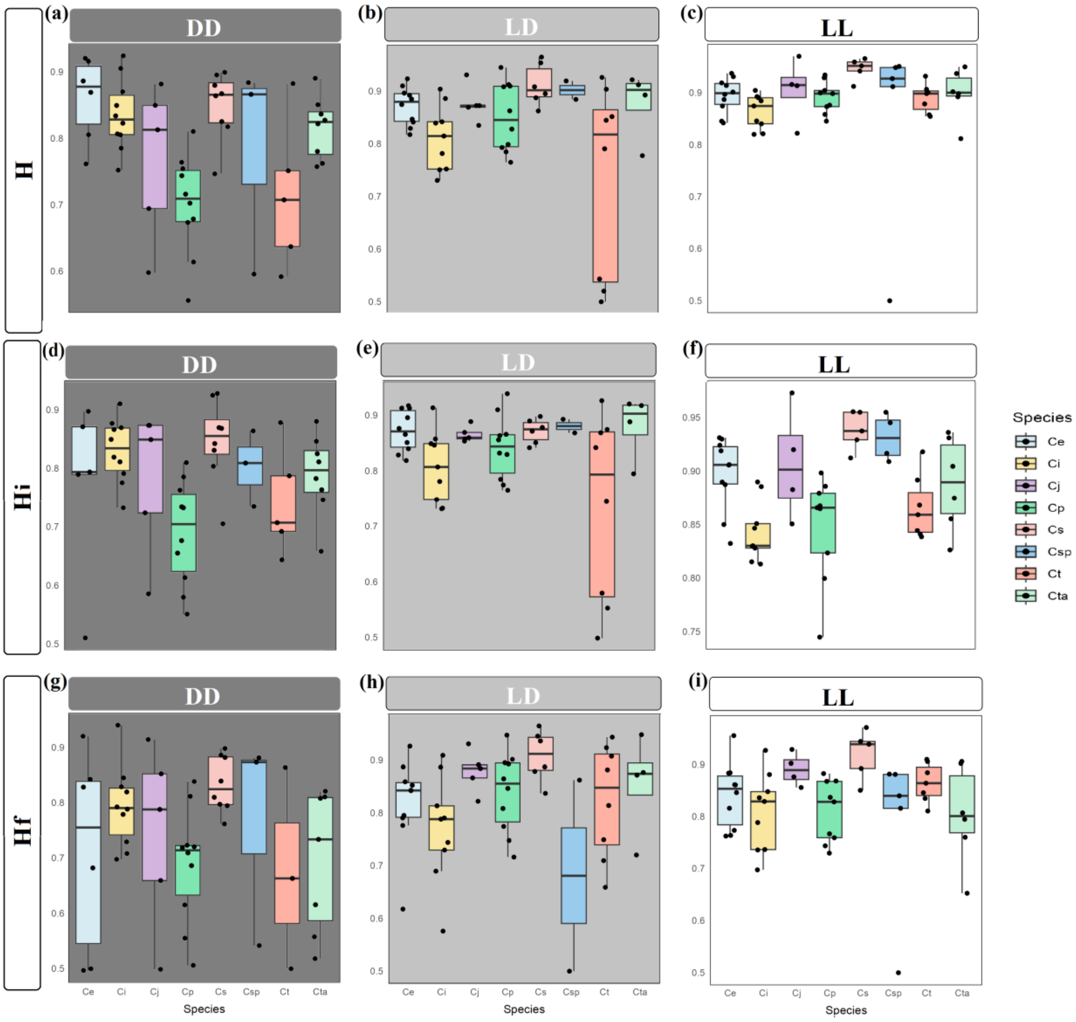

Fig.7. Variation of the Hurst coefficient data compared to the DD, LD, and LL treatments, on the first day of recording (Hi), on the third day of recording (Hf), and for the three consecutive days sampled (H).

Cta = *C. taboa*; Ct = *C. troglobius*; Csp = *C. spelaeus*; Cs = *C.santanensis*; Cp = *C. potiguar*; Cj = *C. jibaossu*; Ci = *C. iuiu*; Ce = *C. eleonorae*

Table 2. Variation in the values of Hi, Hf, and H among troglophilic and troglobitic species subjected to different lighting conditions (DD, LL, and LD).

	Treatment	F(value) / Chi-Square(χ^2)	*p* -value
H	DD	F(7,47) = 4501	0.67e-03
	LL	F(7,47) = 0.997	0.445
	LD	χ^2= 13.06	0.071
Hi	DD	F(7,46) = 2.711	0.0194
	LL	F(7,46) = 5.987	5.48e-05
	LD	χ^2= 11.416	0.122
Hf	DD	F(7,44) = 1.416	0.223
	LL	F(7,47) = 1.863	0.0974
	LD	χ^2= 11.524	0.117

Discussion

Based on the data provided, it is evident that there was no discernible difference in rhythmic elements between troglophilic and troglobitic species, thus contradicting both of our hypotheses. Moreover, differentiations in rhythmic elements among species were not clearly discernible, suggesting that a more comprehensive understanding might be achieved through individual analysis or when segregated by age and sex, potentially revealing correlations. Additionally, a subset of individuals displayed infradian rhythms, while others exhibited circadian rhythms, making it challenging to draw definitive conclusions regarding the circadian phenotype of these cave-dwelling species (Beale *et al*., 2016).

Upon analyzing the main periods of troglophilic species compared to troglobitic ones, no differentiation in correlation with the degree of regression of time-marking mechanisms was observed, as reported by Trajano *et al*. (2009) on troglobitic fishes *Rhamdia enfurnada* and *Trichomycterus itacarambiensis*.

Bimodality was also not evident in the data concerning intervals or main periods, as observed, for instance, in the troglobitic harvestman *Pachylospeleus strinatii*. In this species, two distinct peaks of activity were noted within 24 hours, despite its rhythm being classified as circadian during the DD treatment in which it was tested (Hoenen & Gnaspini, 1999).

Overall, across all treatments, species displayed asynchrony in their amplitudes and main periods, lacking a clearly defined resting period. The presence of aperiodicity in rhythms, where there is no consistent pattern of repetition within a specific time interval, is a common observation in many troglobitic species (Weber, 1980; Martin & Weber, 1985; Lamprecht & Weber, 1992). In the case of troglophiles, situations may arise where two rhythms are evident in response to distinct light conditions, as seen in the orthopterans *Dolichopoda geniculata* and *Hadenoecus subterraneus* subjected to the DD treatment, where some individuals exhibited circadian rhythms while others displayed ultradian rhythms (Simon, 1973; Pasquali *et al.*, 2005).

The ability of these species to respond to light stimuli has likely been compromised, even though other internal clocks, such as peripheral ones, may still be functional. Prolonged isolation from the natural day-night cycle can lead to significant alterations in the function of circadian clock, potentially resulting in the gradual loss of certain mechanisms that no longer confer a selective advantage to these animals (Cavallari *et al.*, 2011). For instance, Menna-Barreto and Trajano (2015) observed that troglophiles generally do not exhibit well-defined rhythmic patterns, as these animals can move between surface and subterranean habitats, facilitating genetic connectivity between populations in both environments. Moreover, regarding the troglophilic *Charinus* species examined in this study, it remains uncertain whether these arachnids maintain constant mobility to areas where light could act as a zeitgeber.

Although light entrainment can be observed in an environment devoid of light cycles, this peculiarity can be interpreted as residual photosensitivity capable of influencing circadian oscillators in specimens synchronized with the main period (Soriano-Morales *et al.*, 2013). Furthermore, it is noteworthy that a significant portion of the *Charinus* specimens displayed both circadian and infradian rhythms, exhibiting some variability in activity patterns, including aperiodicity. This diversity in activity profiles suggests the potential existence of non-photic zeitgebers in environments lacking direct sunlight exposure (Stringer & Rochow, 1997).

Since it might be expected that most individuals within a species would exhibit the same activity pattern, the observed variation in activity among *Charinus* specimens was intriguing. Almost all species displayed both nocturnal and diurnal individuals, except for *C. santanensis*. Given that caves are recognized as oligotrophic environments, this individual variation in activity periods could potentially reduce intraspecific competition for food resources, thereby representing an adaptive strategy within the different species of *Charinus* to maintain population sizes at minimum levels. Competition for food resources within caves can significantly affect population size (Lamprecht, 1978) and resource allocation (Griffith, 1993), influencing foraging efficiency and inducing behavioral changes (Boulay, 2010). Moreover, the presence of other species may further constrain the niche occupied by *Charinus*, potentially driving adaptation to the habitat (Culver, 1970).

In this context, dietary needs may act as “masking factors”, influencing the activity period, as demonstrated by the examined animals (Gnaspini *et al.*, 2003). Additionally, a similar pattern was observed in the harvestman *P. strinatii*, suggesting that food scarcity may have prompted the species to search for food more frequently and intensely. This could have led to the duplication of the original nocturnal activity expression, resulting in the bimodality observed in the study *in situ* by Hoenen and Gnaspini (1999).

While it is understood that many troglobites have lost their temporal organization, particularly concerning circadian rhythms, some species appear to maintain a circadian cycle (*e.g.*, Friedrich *et al.*, 2011). However, in some cases, the manifestation of this rhythm can be highly variable. For instance, in studies with the troglobitic decapod *Orconectes pellucidus*, a diversity of rhythmic patterns was observed, as evidenced by locomotor activity, respiratory activity, and reproductive cycle. In terms of locomotor activity, animals exhibiting arrhythmic, circadian, and infradian patterns were observed under constant light conditions (LL), while under alternating light and darkness conditions (LD), the pattern was arrhythmic and circadian (Park, Roberts & Harris, 1941; Brown, 1961). However, in additional studies, the circadian pattern was predominantly observed for the same treatments with the species *O. pellucidus* (Jegla & Poulson, 1968, 1970).

Analyses of the Hurst exponent under constant light conditions (LL) revealed a trend in rhythm over the three days of observation and also on the first day, with significant differences in the distribution of values among species in different treatments ($p < 0.05$). These findings align with prior research highlighting the variability in the expression of rhythms among the studied organisms: *Glyphiulus cavernicolus* and *Orchestia gammarellus* (Koilraj *et al*., 2000; Rossano, Morgan & Scapini, 2008).

However, regarding the level of synchronization (IS) under alternating light and dark conditions (LD), the values did not indicate evidence of synchronization with the circadian rhythm, revealing considerable variability in individual data. This variability has been noted in other studies involving cave animals (Lamprecht & Weber, 1979; Pasquali *et al.,* 2007; Auletta, Inguscio & Pasquali, 2017), suggesting that individual differences in response to the presence of the light-dark cycle may lead to irregularities in circadian rhythms.

Conclusions

The species of *Charinus* inhabiting caves, subjected to experimental treatments of constant light (LL), constant darkness (DD), and alternating light and darkness (LD) cycles in this study, did not exhibit clear distinctions in their rhythms between troglophilic and troglobitic species. These results underscore the complex dynamics involved in the determination and evolution of circadian rhythms within these populations. Additionally, individual variations in activity patterns were observed, indicating a diversity in the behavioral profiles of these species. Competition for food resources, likely intensified by the oligotrophic constraints observed in caves, emerges as a potential factor shaping these patterns. While some organisms maintain a circadian cycle, its expression proves to be variable, contradicting previous expectations and highlighting the presence of infradian rhythms. This challenges previous research on chronobiological behavior in caves, especially among troglobites. Furthermore, the analyses underscore the importance of adopting a more individualized approach and considering non-photic zeitgebers for a comprehensive and refined understanding of rhythms in this group of organisms.

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Author contributions

RLF, MSS, and PES conceived this study. PES carried out the ex situ sampling and maintenance of specimens. BSBG and PES participated in the data analysis. PES led the writing. RLF collaborated in the discussion during writing, contributed to the elaboration of the in situ species plate, and reviewed the paper.

Conflict of interest

All authors declare no conflict of interest regarding this paper or any information generated or extracted from it.

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APPENDIX I

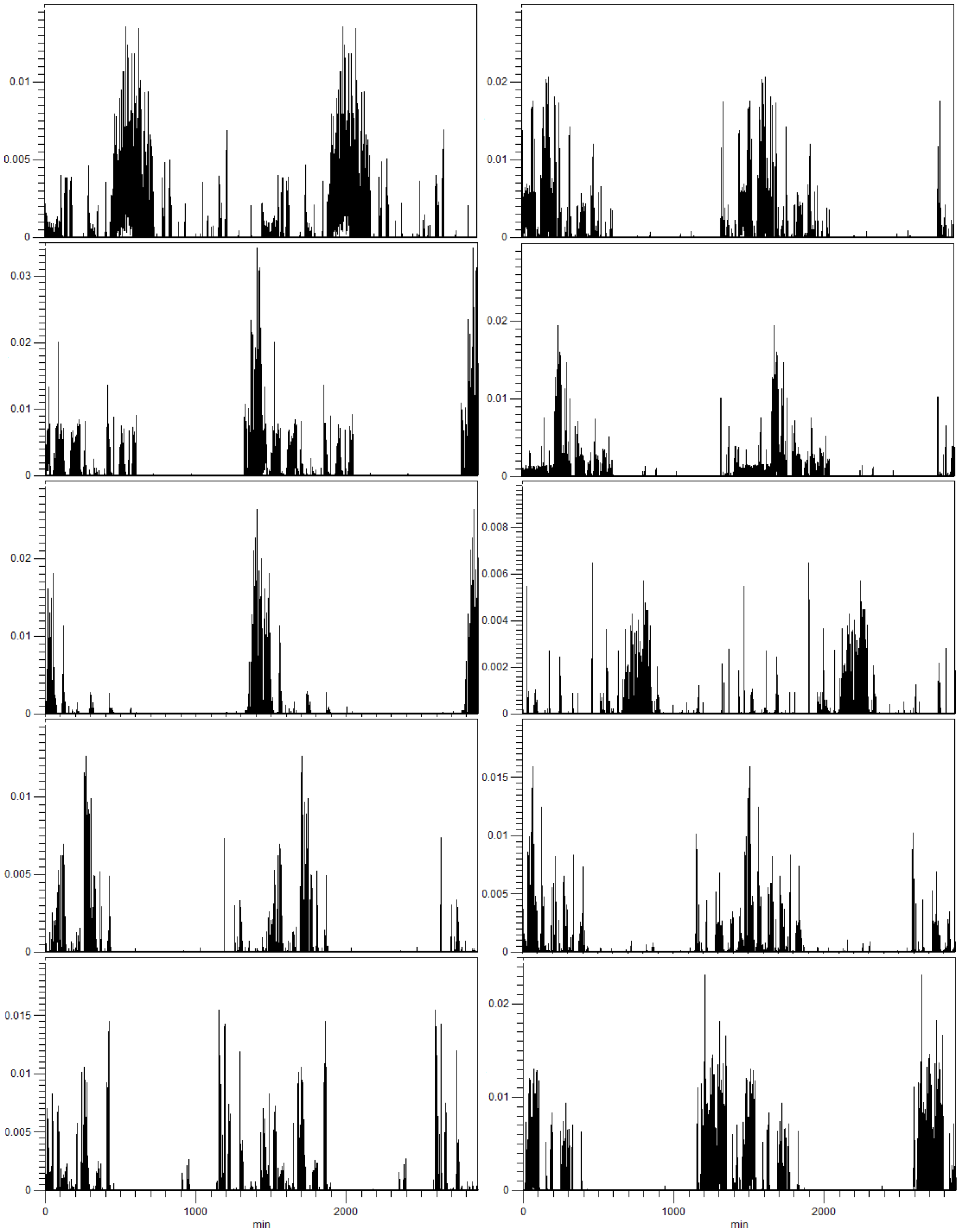

Fig. 1. Medium wave doubly plotted relative to the record of three consecutive days under LD treatment showing the locomotor/rest activity of each individual of the species *Charinus eleonorae*.

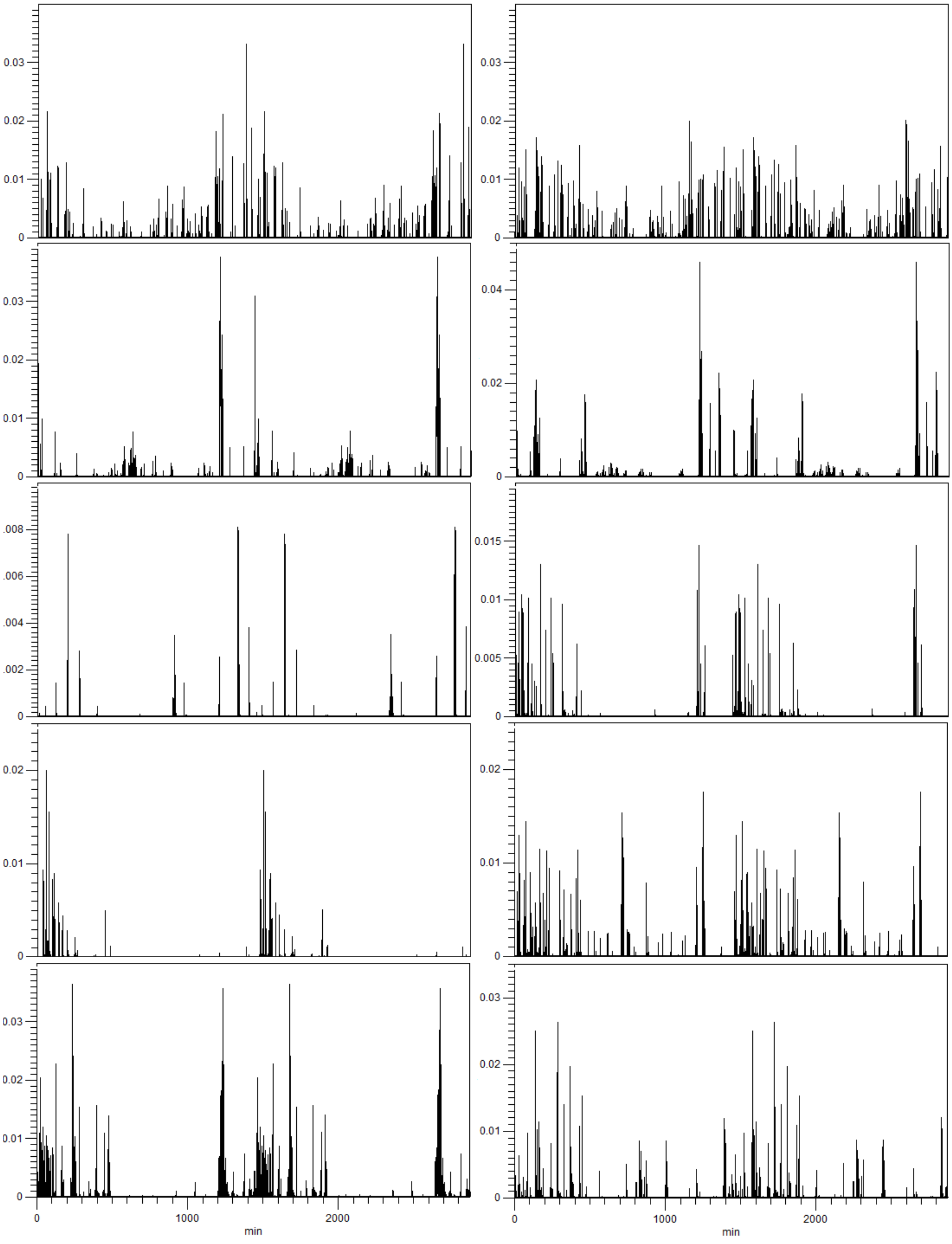

Fig. 2. Medium wave doubly plotted relative to the record of three consecutive days under LD treatment showing the locomotor/rest activity of each individual of the species *Charinus iuiu*.

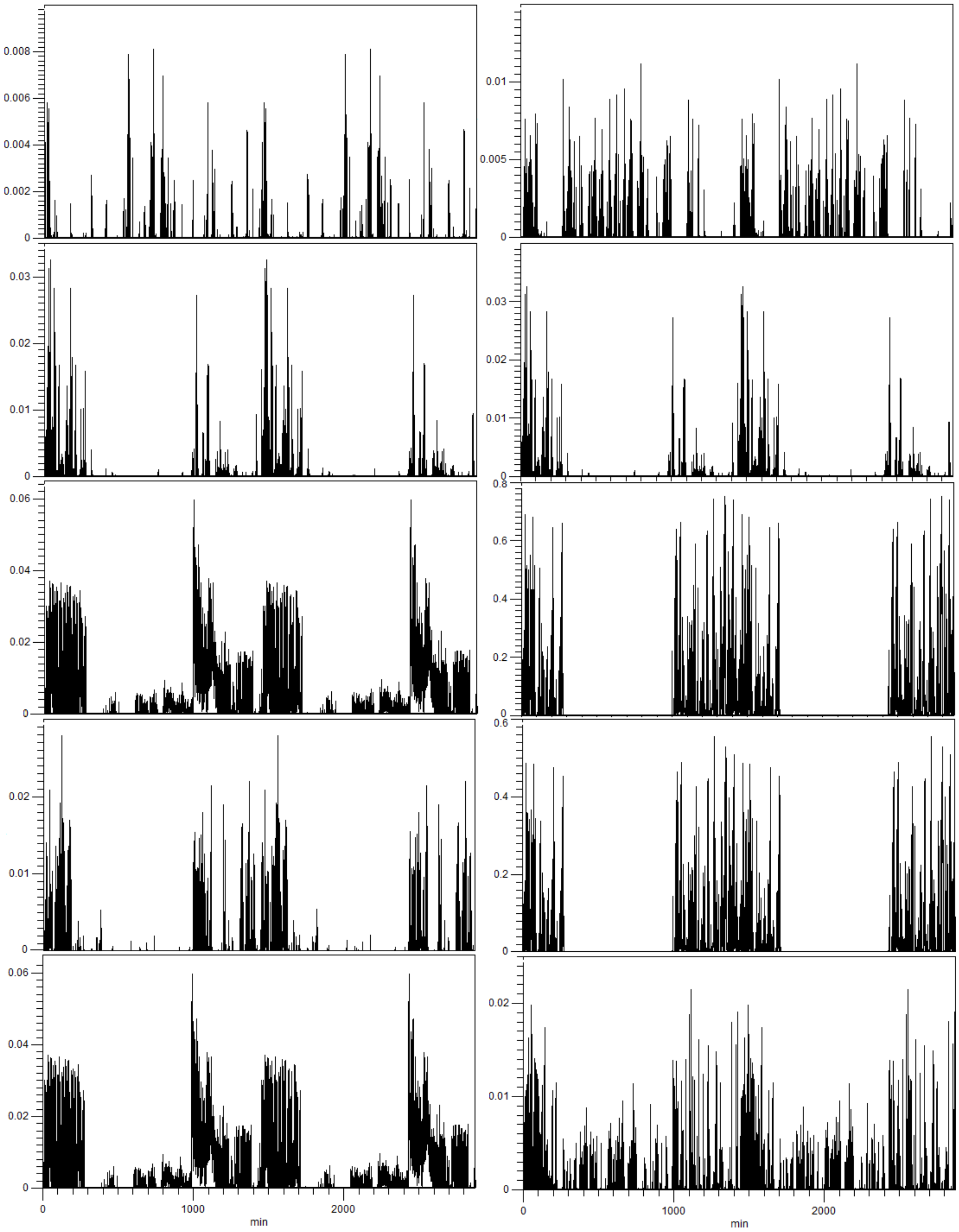

Fig. 3. Medium wave doubly plotted relative to the record of three consecutive days under LD treatment showing the locomotor/rest activity of each individual of the species *Charinus potiguar*.

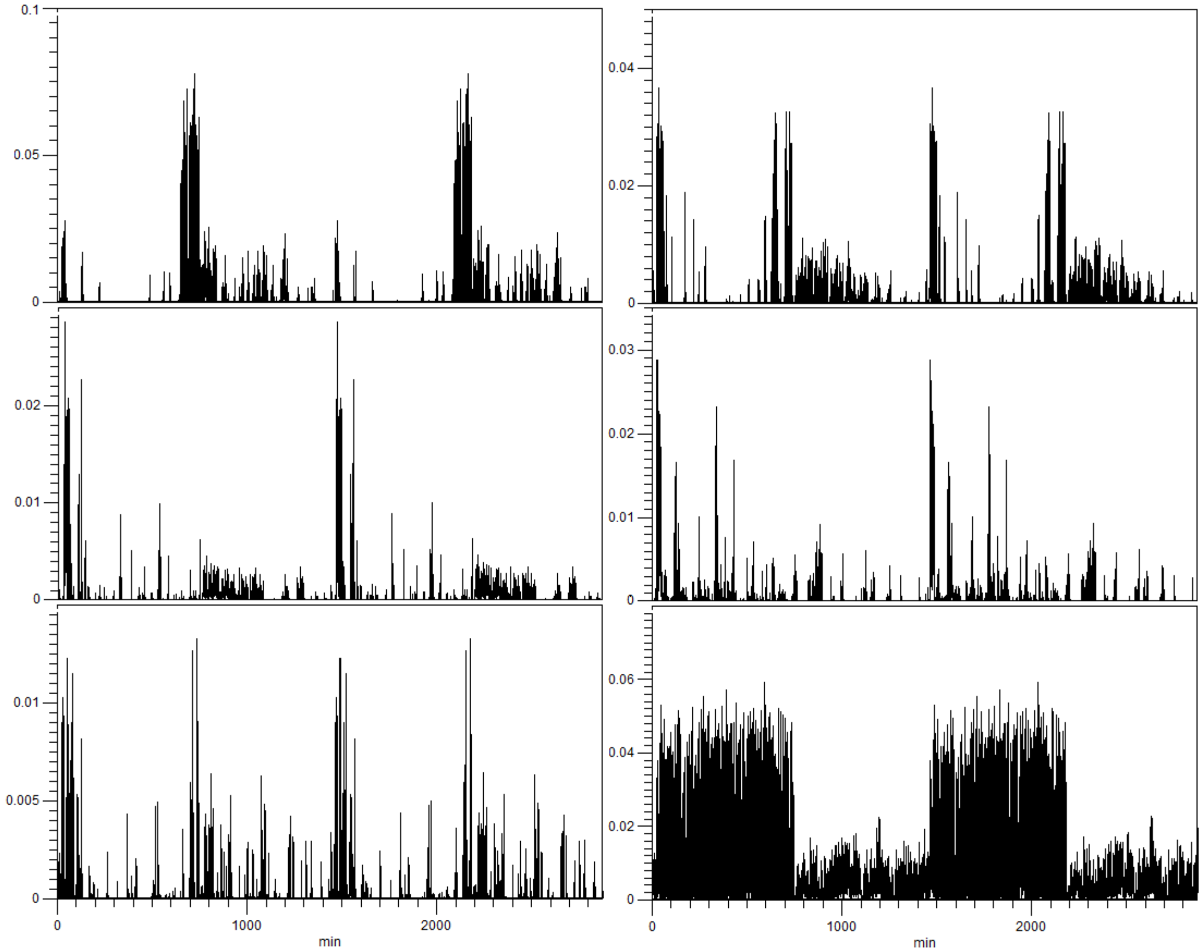

Fig. 4. Medium wave doubly plotted relative to the record of three consecutive days under LD treatment showing the locomotor/rest activity of each individual of the species *Charinus santanensis*.

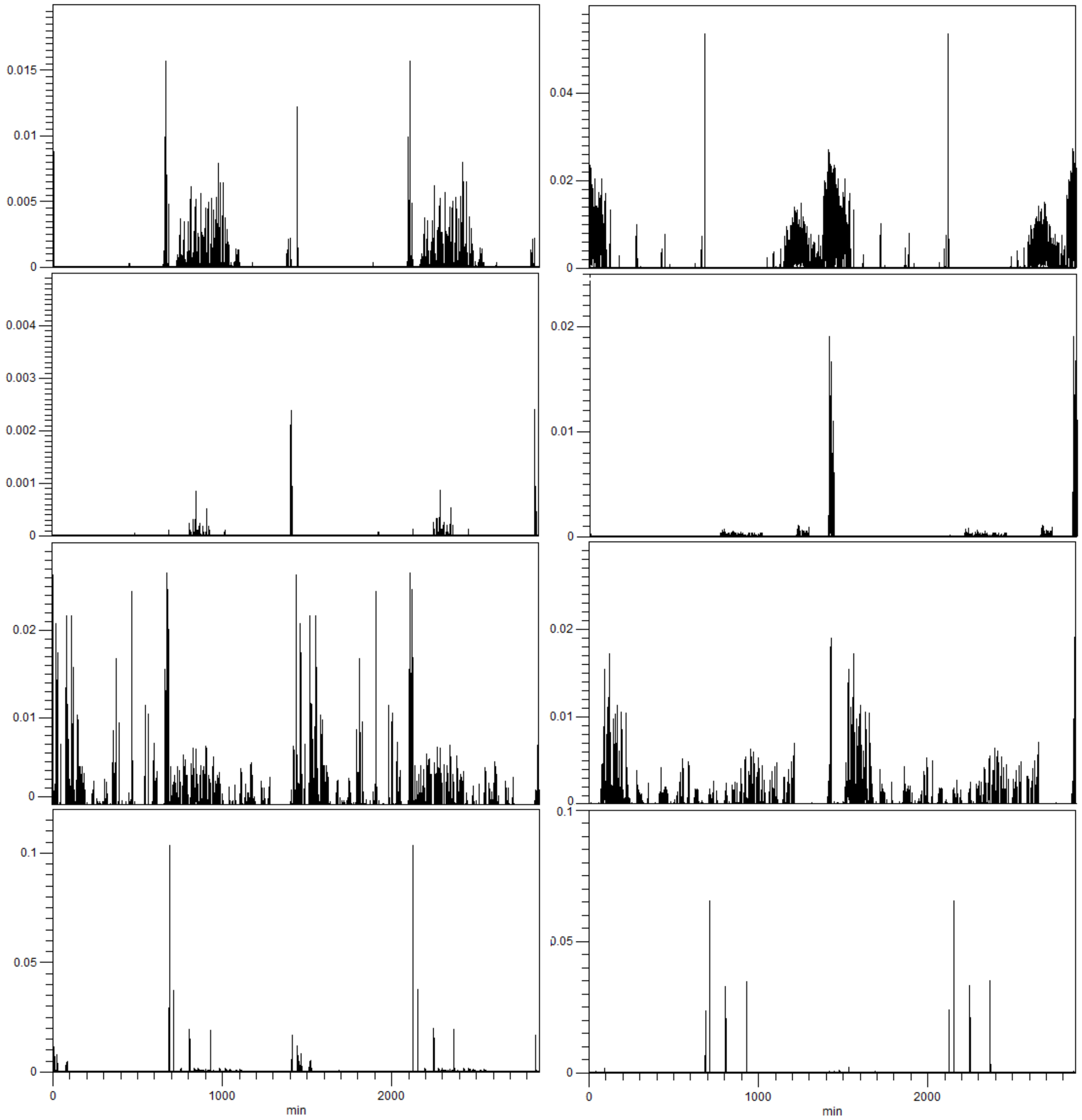

Fig. 5. Medium wave doubly plotted relative to the record of three consecutive days under LD treatment showing the locomotor/rest activity of each individual of the species *Charinus troglobius*.

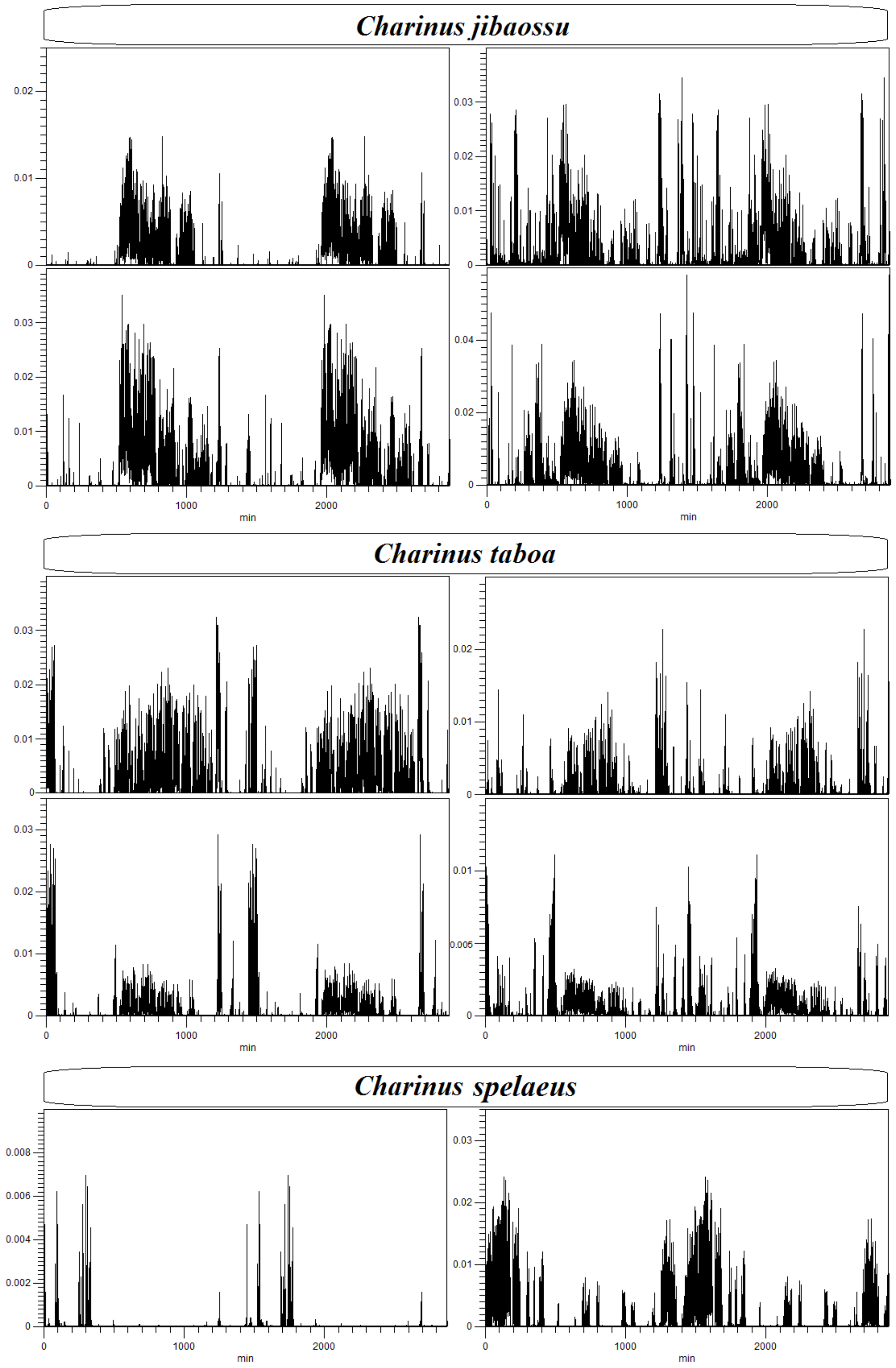

Fig. 5. Medium wave doubly plotted relative to the record of three consecutive days under LD treatment showing the locomotor/rest activity of each individual of the species *Charinus jibaossu, C. taboa and C. spelaeus*.

APPENDIX II

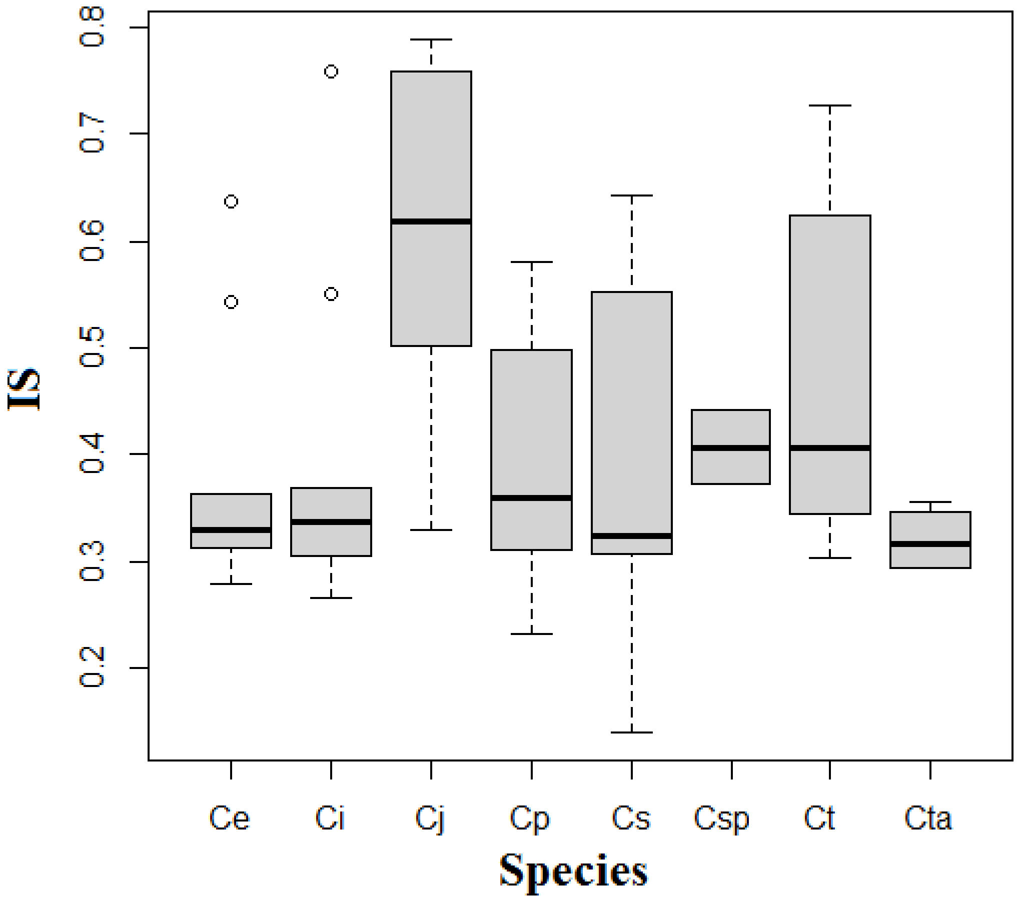

Fig. 1. Variation in Intermediate Stability (IS) values for each *Charinus* species under LD conditions.

Cta = *C. taboa*; Ct = *C. troglobius*; Csp = *C. spelaeus*; Cs = *C. santanensis*; Cp = *C. potiguar*; Cj = *C. jibaossu*; Ci = *C. iuiu*; Ce = *C. eleonorae*.

GLOSSÁRIO

ACOPLAMENTO	Coupling	Coordenação de processos biológicos com eventos ambientais ou ciclos naturais, como o ciclo dia-noite.
APERIÓDICO	Aperiodic	Descreve ritmos biológicos ou fenômenos que não seguem um padrão claro ou previsível ao longo do tempo.
ARRASTAMENTO	Entrainment	O ajuste temporal de um ritmo com outro ritmo refere-se ao processo de sincronização de organismos aos ciclos ambientais.
ARRTMICO	Arrythmic	Padrões biológicos que não exibem uma repetição regular ao longo do tempo.
ASSINCRONIA	Asynchrony	Refere-se à falta de sincronização entre os ritmos biológicos de um organismo e os ciclos ambientais.
ATIVIDADE LOCOMOTORA	Locomotor activity	Movimento físico de um organismo. Na cronobiologia, é estudada em relação aos ritmos biológicos e pode influenciar padrões de atividade e repouso ao longo do dia e da noite.
CARÁTER ENDÓGENO	Endogenous character	A natureza intrínseca. Refere-se à capacidade de um ritmo biológico ser gerado internamente pelo organismo, sem depender de estímulos externos.
CÍCLICO	Cyclic	Descreve padrões biológicos que se repetem regularmente ao longo do tempo, seguindo um ciclo previsível (ex: ciclo dia/noite).

CICLO	Cycle	Intervalo temporal durante o qual uma sequência de eventos ocorre. Em outras palavras, são padrões biológicos recorrentes que seguem uma sequência previsível ao longo do tempo.
CICLO CLARO/ESCURO	Light/dark cycle	Refere-se à alternância entre períodos de luz e escuridão no ambiente, influenciando os ritmos biológicos dos organismos.
CIRCADIANO	Circadian	Refere-se a ritmos com período de aproximadamente 24 horas, como o ciclo sono-vigília.
COMPORTAMENTO CIRCADIANO	Circadian behavior	Refere-se aos padrões comportamentais que seguem um ciclo de ± 24 horas.
DIA	Day	Termo utilizado para se referir tanto ao ciclo dia/noite quanto para a fase clara desse ciclo.
DIURNO	Diurnal/daytime	Eventos que têm uma tendência a ocorrer durante o dia, no caso, a frequência da atividade locomotora. Fase clara do ciclo claro/escuro.
EPÍGEO	Epigeal/epigean	Organismos que ocorrem na superfície.
EX SITU	*Ex situ*	Práticas realizadas fora do habitat natural de uma espécie.
FASE	Phase	Indica um ponto específico em um ciclo periódico, como o ciclo circadiano, onde ocorre uma atividade.

FASE DE CLARO	Light phase	Refere-se em relação ao ciclo claro/escuro, a fase clara, o dia.
FASE DE CLARO	Dark phase	Refere-se em relação ao ciclo claro/escuro, a fase de escuro, a noite.
FOTOPERÍODO	Photoperiod	Refere-se à duração da exposição à luz e escuridão em um ciclo diário de 24 horas.
FREQUÊNCIA	Frequency	Refere-se à taxa de repetição de um padrão ao longo do tempo.
HIPÓGEO	Hypogeal/ hypogean	Organismos que ocorrem no subterrâneo.
IN SITU	*In situ*	Práticas realizadas no habitat natural de uma espécie.
INFRADIANO	Infradian	Refere-se a ritmos biológicos com períodos ou periodicidade maiores que 28 horas.
LIVRE-CURSO	Free-running	Refere-se ao comportamento de um ritmo quando não está mais sincronizado com os ciclos ambientais externos, como luz e escuridão, ou quando não está mais sujeito a influências ambientais.
MASCARAMENTO	Masking	Processo de alteração da expressão de um ritmo biológico.

NOTURNO	Nocturnal/nighttime	Eventos que têm uma tendência a ocorrer durante a noite. Fase escura do ciclo claro/escuro.
OSCILAÇÃO	Oscillation	Mudança do estado de uma variável ao longo do tempo.
OSCILADORES	Oscillators	Sistemas biológicos que geram ritmos biológicos regulares, fundamentais para a regulação de processos fisiológicos e comportamentais.
PADRÃO	Pattern	Conjunto previsível de comportamentos ou eventos biológicos que ocorrem em ciclos específicos ao longo do tempo. Ex: padrões de ritmo, padrões comportamentais.
PERÍODO	Period	São sistemas biológicos que geram e controlam ritmos biológicos, como os ritmos circadianos, em organismos.
PERÍODO EM LIVRE-CURSO	Free-running period	Duração de um ciclo em livre-curso. Indica um estágio onde um ritmo não está mais alinhado com os ciclos ambientais externos, seguindo apenas seu ciclo interno.
PERÍODO PRINCIPAL	Main period	Refere-se ao intervalo de tempo que caracteriza o ritmo biológico dominante de um organismo, onde ocorre a maior parte da atividade ou eventos fisiológicos associados a esse ritmo.
PLASTICIDADE	Plasticity	Capacidade dos ritmos de se adaptarem a mudanças no ambiente ou em condições experimentais.

RELÓGIO BIOLÓGICO	Biological clock	Sistema interno que regula ritmos biológicos e comportamentais em ciclos regulares atuando como mecanismos temporizadores e permitindo aos organismos antecipar e se adaptar a mudanças ambientais.
RELÓGIO PERIFÉRICO	Peripheral clock	Mecanismo presente em órgãos e tecidos fora do cérebro que controla ritmos biológicos locais, promovendo a homeostase e a regulação do metabolismo.
RITMICIDADE	Rhythmicity	Qualidade dos ritmos biológicos que apresentam uma repetição regular ao longo do tempo.
RITMO	Rhythm	Refere-se a uma sequência regular e previsível de eventos ou atividades que ocorrem em ciclos repetitivos ao longo do tempo.
RITMO BIOLÓGICO	Biological rhythm	Resultado da interação entre mecanismos internos (endógenos) e sincronizadores externos (exógenos).
SINCRONIZAÇÃO	Synchronization	Alinhamento dos ritmos biológicos de um organismo com os ciclos ambientais ou ritmos internos, mantendo uma relação estável de fase entre os ciclos, que pode ser alcançada por meio de arrastamento ou mascaramento.
TROGLÓBIO	Troglobite	Organismos adaptados exclusivamente a ambientes subterrâneos, caracterizados por condições de escuridão e isolamento.

TROGLÓFILO	Troglophile	Organismos adaptados que habitam tanto ambientes superficiais quanto subterrâneos.
TROGLOMORFISMOS	Troglomorphisms	Características morfológicas adaptativas de organismos que habitam ambientes subterrâneos, incluindo ausência de pigmentação, redução ou ausência do sistema visual e alongamento de apêndices.
TROGLÓXENO	Trogloxen	Organismos que ocasionalmente frequentam ambientes subterrâneos, como cavernas, mas que dependem regularmente da vida na superfície.
ULTRADIANO	Ultradian	Periodicidade menor que 20 horas.
VARIABILIDADE	Variability	Flutuações ou mudanças na magnitude ou na frequência dos ritmos biológicos ao longo do tempo. A compreensão da variabilidade dos ritmos biológicos é importante para entender a plasticidade e a adaptabilidade dos organismos a diferentes condições ambientais.
ZEITGEBER	Zeitgeber	Expressão alemã para “doador do tempo”, se referindo a estímulos externos que sincronizam e ajustam os ritmos biológicos endógenos de um organismo.