Subterranean Biology 20: 1–13 (2016)
doi: 10.3897/subtbiol.20.10010
http://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Incipient regressive evolution of the circadian rhythms of a cave amphipod

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

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

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

Academic editor: *O. Moldovan* | Received 26 July 2016 | Accepted 14 September 2016 | Published 7 October 2016

http://zoobank.org/BF313AAB-6F45-4E9E-95BE-2013BDDBA593

Citation: Espinasa L, Collins E, Finocchiaro A, Kopp J, Robinson J, Rutkowski J (2016) Incipient regressive evolution of the circadian rhythms of a cave amphipod. Subterranean Biology 20: 1–13. doi: 10.3897/subtbiol.20.10010

Abstract

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

Keywords

Stygobromus allegheniensis, Shawangunk, nyctophilia, Ice Caves, Sam's Point Preserve, Clarksville Cave, troglobite, troglobiont, light-entrainment

Introduction

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

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

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

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

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

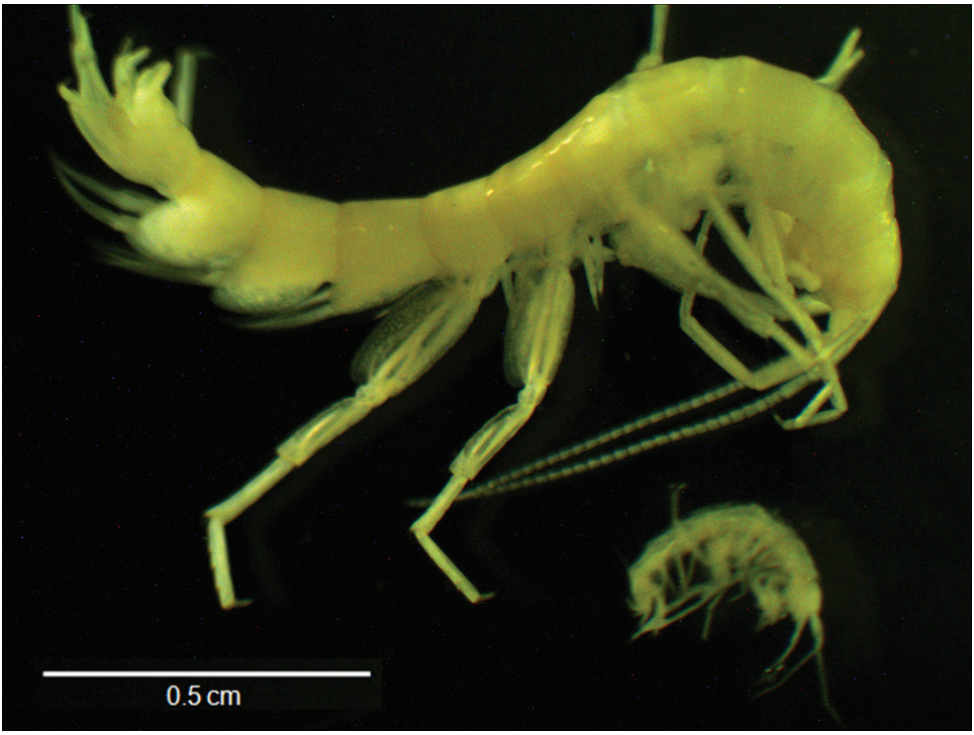

Figure 1. Adult and juvenile specimens of *Stygobromus allegheniensis* from Ice Cave #1 at Sam's Point Preserve. As is typical of cave-adapted organisms, this species is depigmented, has long appendages, and is fully eyeless. Nonetheless, it can detect light and actively avoids it.

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

Methods

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

Field experiments

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

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

Laboratory experiments

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

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

Results

Laboratory experiments

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

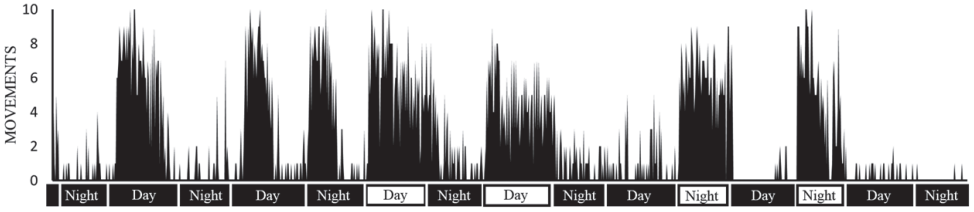

Figure 2. Experimental protocol and representative motor rhythms of one individual. Ice Cave individuals were subjected in the laboratory to the following conditions: Five half-cycles of darkness, followed by two cycles of light/dark during normal day/night schedules, followed by two cycles of dark/light during reverse day/night schedules, followed by a half-cycle of darkness. Black boxes indicate dark conditions while white boxes represent illuminated conditions. Movements were evaluated for each 10-minute period.

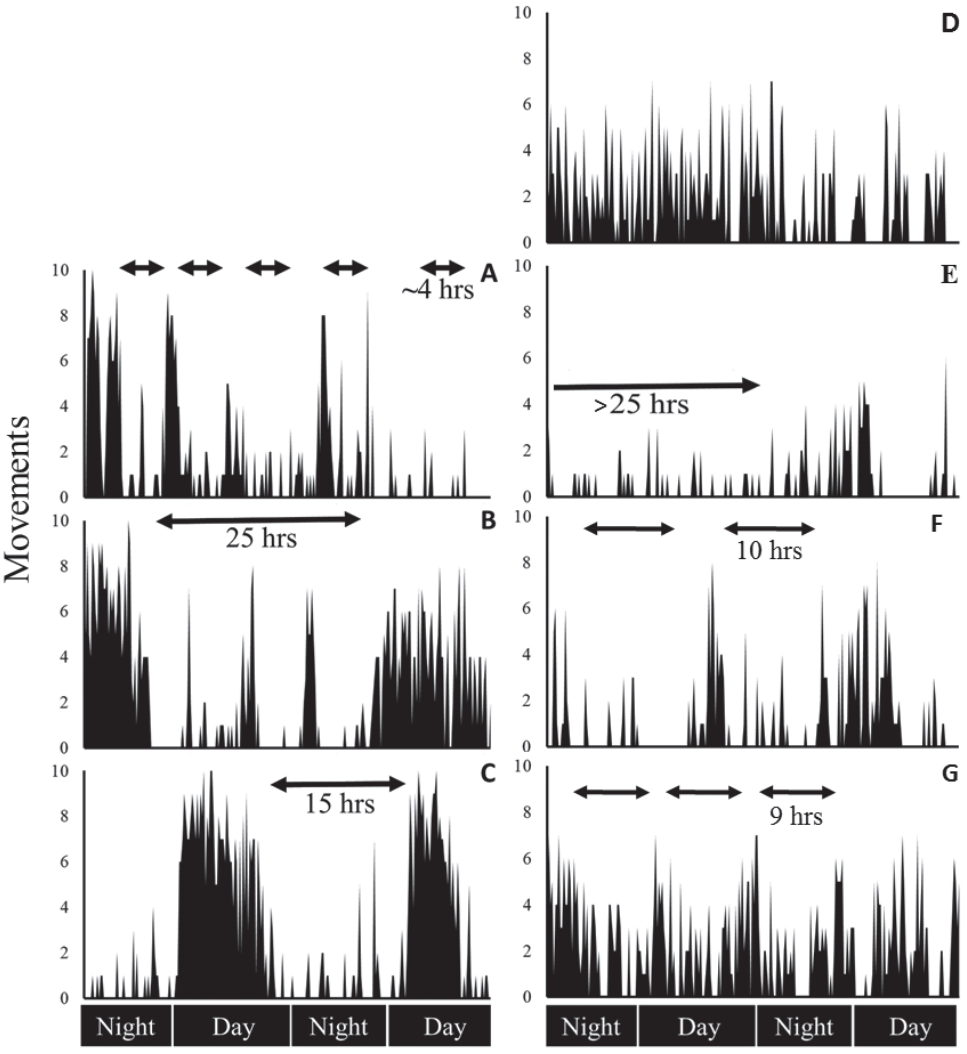

Figure 3. Variability in motor rhythms while in continuous darkness in three Ice Cave individuals (**A–C**) and four Clarksville Cave individuals (**D–G**) tested in the laboratory. Black boxes indicate periods while in darkness.

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

The periods of rest were neither synchronized among the individuals, nor synchronized to day and night schedules. For example, the individual depicted in Figure 2 was out of synchronization with the day/night cycles despite having a cycle close to 24 hrs. In this individual, during the first 60 hours of continuous darkness the first two periods of activity coincided with daytime hours, but by the third period it had

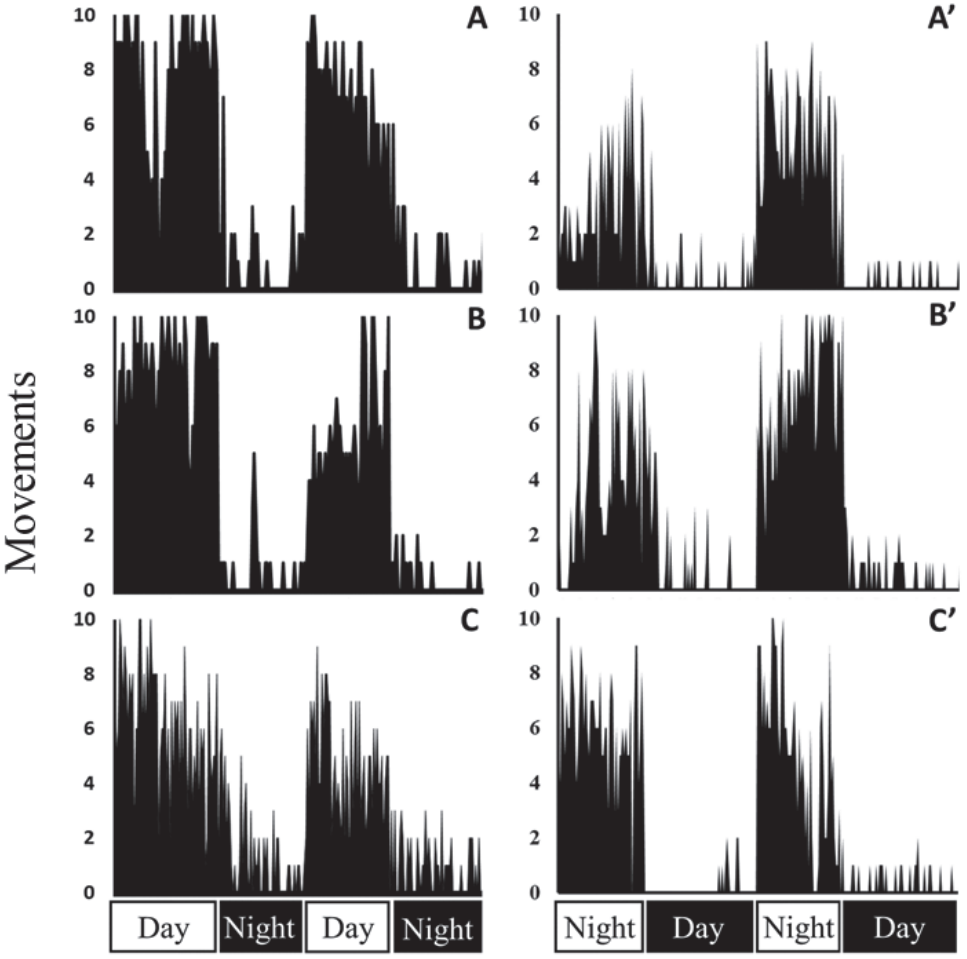

Figure 4. Motor activity followed periods of light or darkness regardless of the time of the day. Individuals on the left (**A–C**) are the same as individuals on the right (**A'–C'**). Black boxes indicate periods while in darkness and white boxes indicate illuminated conditions.

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

When the specimens were subjected to LD 14:10 cycles, activity/rest patterns matched illumination (Figure 4), regardless of the time of day or night. Specimens on a LD cycle where light coincided with daytime and dark coincided with nighttime (Figure 4A–C) or on a DL cycle where dark coincided with daytime and light coincided with nighttime (Figure 4D–F), they followed the illumination patterns and not the time of the day. Statistical analysis also support that periods of activity versus rest were not randomly distributed through time ($P<0.001$ for all individuals), but instead were clustered. While this pattern first gives the appearance of motor rhythms

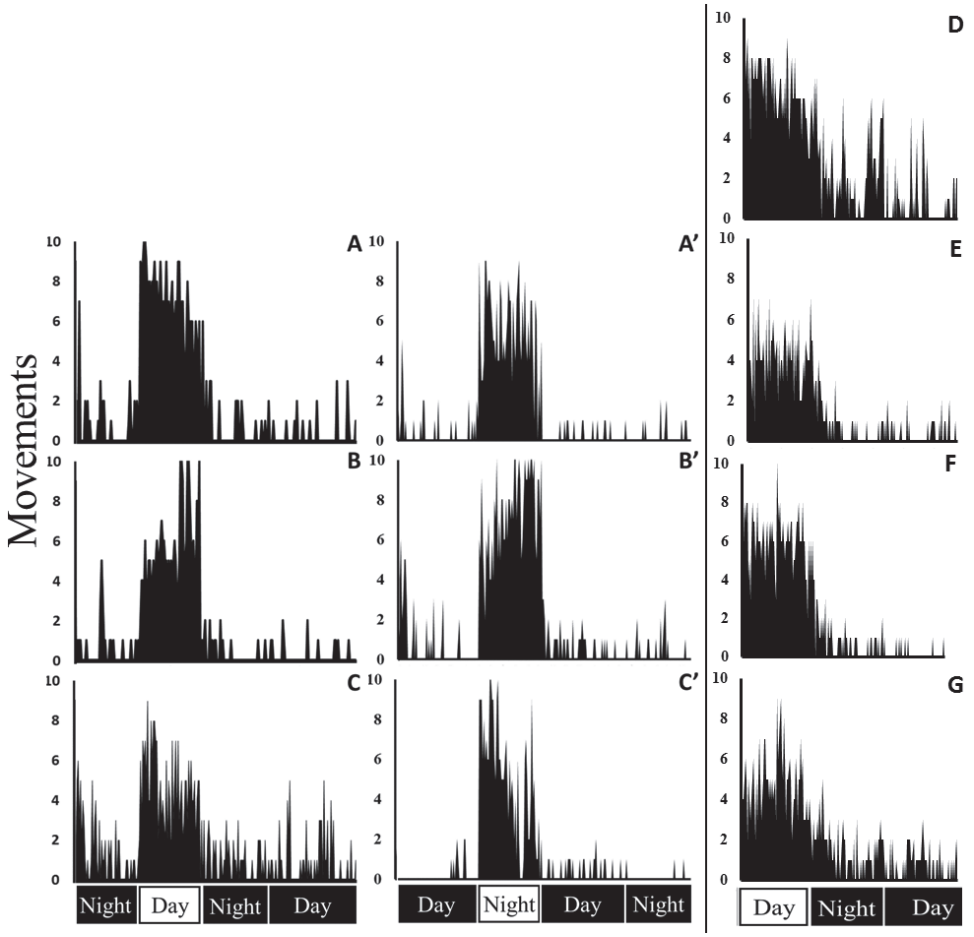

Figure 5. Entrainment by light is apparently not functioning in the Ice Cave (**A–C** and **A'–C'**) and Clarksville Cave (**D–G**) populations. In *S. allegheniensis*, the second dark period lacks the anticipation and synchronization of a period of activity, which is a hallmark of organisms possessing a light-entrained circadian rhythm. Black boxes indicate periods while in darkness and white boxes indicate illuminated conditions.

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

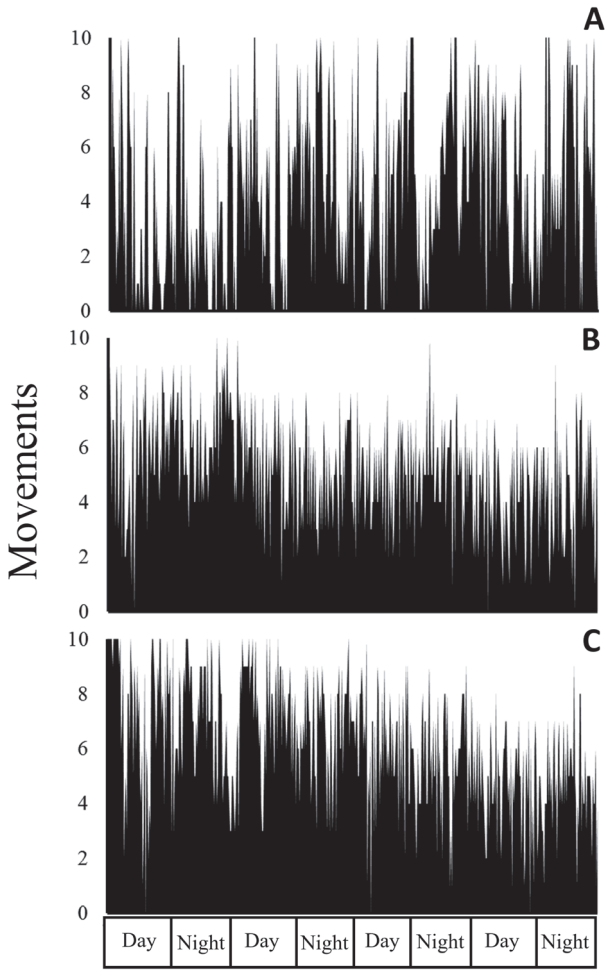

Figure 6. *Stygobromus allegheniensis* has continuous light avoidance behavior which does not appear to follow circadian rhythmicity. White boxes indicate illuminated conditions.

Field experiments

Experiments performed directly in Clarksville Cave and in the Ice Cave confirmed that in their natural environments, specimens behaved similarly in both the laboratory and the natural setting. While in continuous darkness, the specimens' periods of activity and rest did not follow a circadian rhythm (Figure 7). The length of the periods of rest varied greatly among individuals. For most individuals the rest periods are intermittently interrupted by short bouts of activity making it difficult to demarcate periodicity. In only one of the six individuals is there an apparent rest period of 12 hours (Figure 7E). The periods of rest were not synchronized among the individuals. Furthermore, the individual with the 12 hour rest (Figure 7E), periods of activity were not synchronized to day and night schedules.

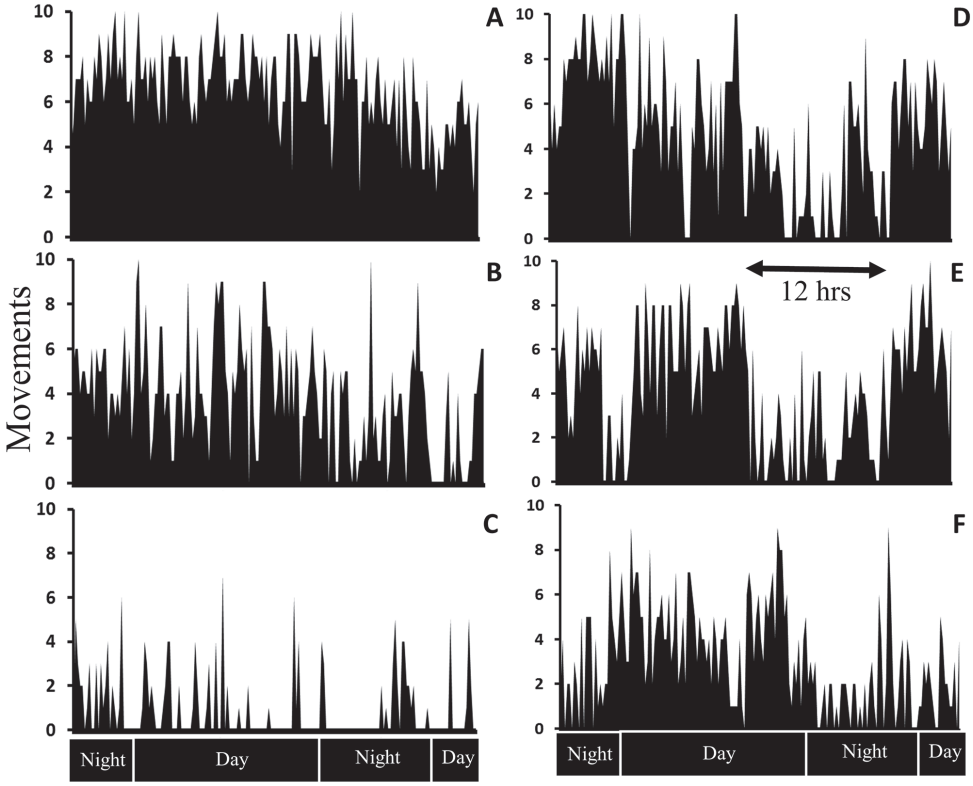

Figure 7. Specimens from Clarksville Cave (**A–C**) and the Ice Cave (**D–F**) studied in the natural environment of the cave. Under continuous darkness, most specimens had periods of activity with no clear indication of periodicity. Only in one of them (**E**) there was an apparent 12 hour rest period. Black boxes indicate periods while in darkness.

Discussion

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

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

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

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

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

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

Conclusions

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

Acknowledgments

The research permit for the Ice Cave was issued by the science staff at the Palisades Interstate Park Commission, Jesse Jaycox and Ed McGowan. We would like to thank the staff at the Sam's Point Area of Minnewaska State Park Preserve for their support, with special mention to Henry Alicandri. We would like to thank the Northeastern Cave Conservancy for granting us a permit to conduct research in Clarksville Cave and to the managers of Clarksville Cave Mike Chu, Chuck Porter and Thom Engel for permission to visit the cave. Bastien Dal Farra, Elsah Epstein, Abigail Descoteaux, Jae Wong, Michael Magin, and students of the Fall 2015 Field Biology (BIOL192) course helped in the field and/or analyzing the recorded videos. Partial support for the project came from the School of Science at Marist College.

References

Beale AD, Whitmore D (2015) Daily Rhythms in a Timeless Environment: Circadian Clocks in *Astyanax mexicanus*. Biology and Evolution of the Mexican Cavefish, 309–303.

Bejder L, Hall BK (2002) Limbs in whales and limblessness in other vertebrates: mechanisms of evolutionary and developmental transformation and loss. Evolutionary Development 4: 445–458. doi: 10.1046/j.1525-142X.2002.02033.x

Cahill A, Kavanagh A, McCahill A, Scott A, Espinasa L (2015) Phylogenetic Analysis of Several New Populations of *Stygobromus allegheniensis* (Allegheny Cave Amphipod) in the Ice Caves of the Shawangunk Ridge, NY. The Northeast Natural History Conference. Springfield, MA.

Chen Y, Zhang Y, Jiang T, Barlow AJ, St Amand TR, Hu Y, Heaney S, Francis-West P, Chuong C, Maas R (2000) Conservation of early odontogenic signaling pathways in Aves. Developmental Biology 97: 10044–10049. doi: 10.1073/pnas.160245097

Espinasa L, Jeffery WR (2006) Conservation of retinal circadian rhythms during cavefish eye degeneration. Evolution and Development 8(1): 16–22. doi: 10.1111/j.1525-142X.2006.05071.x

Espinasa L, McCahill A, Kavanagh A, Espinasa J, Scott AM, Cahill A (2015) A troglobitic amphipod in the Ice Caves of the Shawangunk Ridge: Behavior and resistance to freezing. Subterranean Biology 15: 95. doi: 10.3897/subtbiol.15.4733

Holsinger JR (1967) Systematics, speciation, and distribution of the subterranean amphipod genus *Stygonectes* (Gammaridae). United States National Museum Bulletin 259: 1–176. doi: 10.5479/si.03629236.259.1

Jeffery WR (2001) Cavefish as a model system in evolutionary developmental biology. Developmental Biology 231: 1–12. doi: 10.1006/dbio.2000.0121

Knight LR, Johns T (2015) Auto-ecological studies on *Niphargus aquilex* (Schiödte, 1855) and *Niphargus glenniei* (Spooner, 1952) (Crustacea: Amphipoda: Niphargidae). Cave and Karst Science 42(2): 63–77.

McCauley DW, Hixon E, Jeffery WR (2004) Evolution of pigment cell regression in the cavefish *Astyanax*: a late step in melanogenesis. Evolutionary Development 6: 209–218. doi: 10.1111/j.1525-142X.2004.04026.x

Pasquali V, Sbordoni V (2014) High variability in the expression of circadian rhythms in a cave beetle population. Biological Rhythm Research 45(6): 925–939. doi: 10.1080/09291016.2014.934077

Zar JH (2007) Biostatistical Analysis (5th Edition). Prentice-Hall, Inc. Upper Saddle River, NJ, USA.

Subterranean Biology 20: 15–37 (2016)
doi: 10.3897/subtbiol.20.9334
http://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Are inner cave communities more stable than entrance communities in Lapa Nova show cave?

Thais Giovannini Pellegrini[1], Rodrigo Lopes Ferreira[2]

1 *PPG- Ecologia Aplicada, Departamento de Biologia (DBI), Universidade Federal de Lavras (UFLA), Minas Gerais, CEP: 37200-000, Brasil,* **2** *Centro de Estudos em Biologia Subterrânea, Departamento de Biologia (DBI), Universidade Federal de Lavras (UFLA), Minas Gerais, CEP: 37200-000, Brasil*

Corresponding author: *Thais Giovannini Pellegrini* (thais.g.pellegrini@gmail.com)

Academic editor: *O. Moldovan* | Received 25 May 2016 | Accepted 19 September 2016 | Published 4 November 2016

http://zoobank.org/87F1A346-BEFD-4269-A2E0-46DDA26191BF

Citation: Pellegrini TG, Ferreira RL (2016) Are inner cave communities more stable than entrance communities in Lapa Nova show cave? Subterranean Biology 20: 15–37. doi: 10.3897/subtbiol.20.9334

Abstract

Lapa Nova is a dolomitic Brazilian show cave. Invertebrate fauna registered for this cave is quite rich and abundant. During two intensive surveys in 2009, 24,482 invertebrate individuals belonging to 187 species were sampled. We found 160 species in April sampling, while in September sampling richness was considerably lower, 102 species, with a remarkable species turnover. In this paper the species richness, abundance and species diversity is presented. The Shannon`s diversity index was 2.79 and 2.87 for April and September, respectively. Although one would expect less variations to be found in the deep cave community (when compared to those located near the entrances), due to higher environmental stability, this was not observed at Lapa Nova cave. This "paradox" is probably due to the intense tourism that occurs in the cave, which imposes "instability" in all sectors, not only in nearby entrance areas. Visitation at the cave probably altered the expected natural distribution pattern, imposing a new organization of the communities, driven by the unstable conditions imposed by cave tourism.

Keywords

Cave invertebrates, temporal turnover, beta diversity, tourism impact, Brazil

Introduction

Despite the well know environmental stability in subterranean systems, it is not homogeneous for the whole extension of a cave. With the use of precise monitoring instruments, certain environmental variability can be detected (Romero 2009). Entrance areas in caves present direct atmospheric exchange with the cave (Oliveira et al. 2008, Lobo et al. 2009, Lobo et al. 2015, Tobin et al. 2013). Such areas can be considered as ecotones, situated in boundaries between two relatively homogeneous ecological communities, hypogean and epigean (Prous et al. 2004, Moseley 2009, Prous et al. 2015). Variability in parameters such as temperature is attenuated with increasing distance from the cave entrance (Tobin et al. 2013). Moreover, differences among transitional areas of the caves tend to be more drastic in caves situated in temperate areas (Tobin et al. 2013), when compared to tropical areas.

In spite of the differences in stability among caves situated at different latitudes, the range of environmental instability intensity can also vary in different caves located in a same region. Such differences occur according to their physical conditions, presence of large bat populations, human use, among others (Huppop 2005, Rocha 2013, Lobo et al. 2015). Cave morphology, entrance size and the number of entrances, are important factors influencing stability (Ferreira 2004, Tobin et al 2013). In some cases, larger entrances usually lead to an increase in temperature and humidity seasonal variation, since they impose a more ample connection with the epigean environment (Ferreira 2004, Tobin et al 2013). Accordingly, one would expect less variation to be found in the inner communities when compared to those located near the entrances, due to higher environmental stability. Furthermore, subterranean rivers can also enhance the atmospheric instability inside caves, amplifying the connectivity with the outside (Lobo et al. 2015) and the climatic variations inside caves. In the tropics, huge movements of bat populations leaving and returning to the cave can also cause microclimate changes in subterranean environment (Rocha 2013). Such differences are also detected along sectors in the same cave; daily bat movements are reflected in cave chambers (Rocha 2013). Seasonal stability differences are also reflected in the distribution and abundance patterns of populations associated to the cave environment (Romero 2009). Therefore, seasonal changes exert a considerable influence on cave fauna distribution; many troglobionts and troglophiles are restricted to areas with the most stable temperature and humidity (Tobin et al. 2013) like the deeper passages.

Factors other than microclimate, can determine changes in the distributional patterns of subterranean species. Human use is an important factor that generates instability in the cave environment, especially in cases of high intense tourism. This type of use can modify patterns such as temperature, humidity and speleothem growth (Lobo 2015), and modify distribution patterns of populations inside subterranean environments (e.g. Moldovan et al. 2003; Bernard et al. 2010; Pellegrini and Ferreira 2012; Guil and Trajano 2013, Faille et al. 2015). As an example, high impacts due to intense tourism were detected in Ursilor Cave (Transylvania, Romania), which demonstrated drastic reduction of two endemic cave beetles in sectors with visitation (Moldovan et

al. 2003). The monitoring of their populations conducted before and after tourism implementation showed that such species have altered their distribution and abundance in Ursilor Cave in response to visitation disturbing events. Such species became almost absent in traps installed in the touristic region of the cave, showing that cave visitation is an intensive disturbance for those populations.

Studies investigating distribution of cave fauna have only focused on the cavernicolous species (troglobites) (e.g. Moldovan et al 2003). Troglobites are more frequently found in deeper and more isolated cave areas, with greater environmental stability (Peck 1976, Tobin et al 2013), and in areas of lower impact degree caused by tourists (Moldovan et al 2003, Pellegrini and Ferreira 2012). Although Novak et al. (2012) indicated that terrestrial troglobite fauna is more diverse and randomly distributed in entrance areas (corresponding to the first 10 meters of the cave) and in the shallow subterranean habitat.

Although a recent study indicates the existence of two hotspots of subterranean biodiversity in Brazil (Souza-Silva and Ferreira 2016), Neotropical cave communities are formed mainly by troglophilic organisms (Trajano and Bichuette 2010). In many Brazilian caves, troglobitic species are even unknown (Souza-Silva et al. 2011, Simões et al. 2014, Simões et al. 2015). Thus, studies in Neotropical caves focusing only on troglobite species would eventually neglect most of the species that make up the entire cave community.

However, studies that have accessed temporal and spatial variations of the entire subterranean community are scarce. The understanding of seasonal patterns generates subsidies for subterranean ecosystem conservation and management purposes. As such, the objective of the present work was to identify the alterations undergone by the invertebrate community associated to Lapa Nova, a large dolomitic cave of Minas Gerais state, Brazil, in two different sampling periods, considering two main "compartments": inner communities and those communities associated to areas near entrances. Our main intention was to verify if inner communities are more "stable" than entrance communities considering the mean richness and abundance values in time, and also temporal beta diversity values and species composition variation, which, in theory, are more subject to external variations. It worth stating that in the publication by Pellegrini and Ferreira 2012, changes undergone by the community in an interval of two consecutive months (April and May) due to the cave tourist use were assessed, with a subsequent proposal of an appropriate management plan for the cave. Unlike that study, this present study focuses on community stability in a period of five months, comprising two distinct seasons.

Materials and methods

Study area

The present study was conducted at the Lapa Nova dolomitic show cave, located in Vazante, northwest Minas Gerais state, Brazil (Fig. 2A) (17°59'04.0"S 46°53'26.4"W).

It is the most known and visited cave in northeastern Minas Gerais state, besides being the second most extensive cave of the area with 4,550 meters of linear development (Auler et al. 2001). It is a hypogenic, labyrinthine cave, network shaped, with one big downward entrance and two secondary entrances (Auler et al. 2009).

In order to confirm if the sectors near entrances are more variable when compared to more isolated sectors, we used humidity and temperature data. This data is available at Lapa Nova Management Plan, and it was measured during four days in April (Auler et al. 2009), in the same year that this work was conducted. The external sampling site (TH1), near the principal cave entrance, showed the highest range of temperature and air relative humidity variation from 17.9 to 20.5°C and from 91 to 99% respectively (Figure 1). The entrance-sampling site (TH2) presented a variation from 17.6 to 19.3°C and from 97 to 99%. The two sampling sites in deeper regions (TH3 and TH4) of the cave were considerable stable. One site presented a variation from 18.8 to 19.3°C and air humidity was constant at 99% and the other from 18.9 to 19.8°C and from 97 to 99% (Auler et al. 2009).

Invertebrate sampling

The cave was divided into nine sectors, each corresponding to 1/9 of the total linear extension of Lapa Nova. Three sectors were located in entrance areas (Sectors 1, 4 and 5), the other six were in deeper regions of the cave (Sectors 2, 3, 6, 7, 8 and 9) (Figure 1).

Two five-day field trips were carried out for collecting in the nine sectors, the first was in April, and the other in September, both in 2009. The invertebrate collections were conducted by the same team, composed of five biologists with experience in caving and invertebrates collection, and it was done through manual capture (with the aid of tweezers, brushes and hand nets). Sampling was conduced by visual searching throughout all the accessible places in the cave, prioritizing organic deposits (debris, carcasses, guano, etc.) and microhabitats (spaces under rocks, humid soil, cracks, speleothems, etc.). All the invertebrate species found in the sectors had some of their specimens collected. The organisms observed during the collections were counted and plotted on the cave map according to the methodology proposed by Ferreira (2004), allowing a visualization of the distribution, as well as the relative abundance of the different species found in the cave.

All the collected invertebrates were identified to the lowest taxonomic level possible, using a stereomicroscope. The specimens were separated into morpho-species for determination of species richness. The collected specimens were fixed in 70% alcohol. Subsequently they were deposited in the *Centro de Estudos em Biologia Subterrânea* collection (Zoology Sector / Biology Department, Federal University of Lavras). Troglomorphic species were considered as troglobite, we performed visual searching outside the cave looking for those species under rocks and wood debris and they were not found, indicating that they are restricted to the subterranean environment.

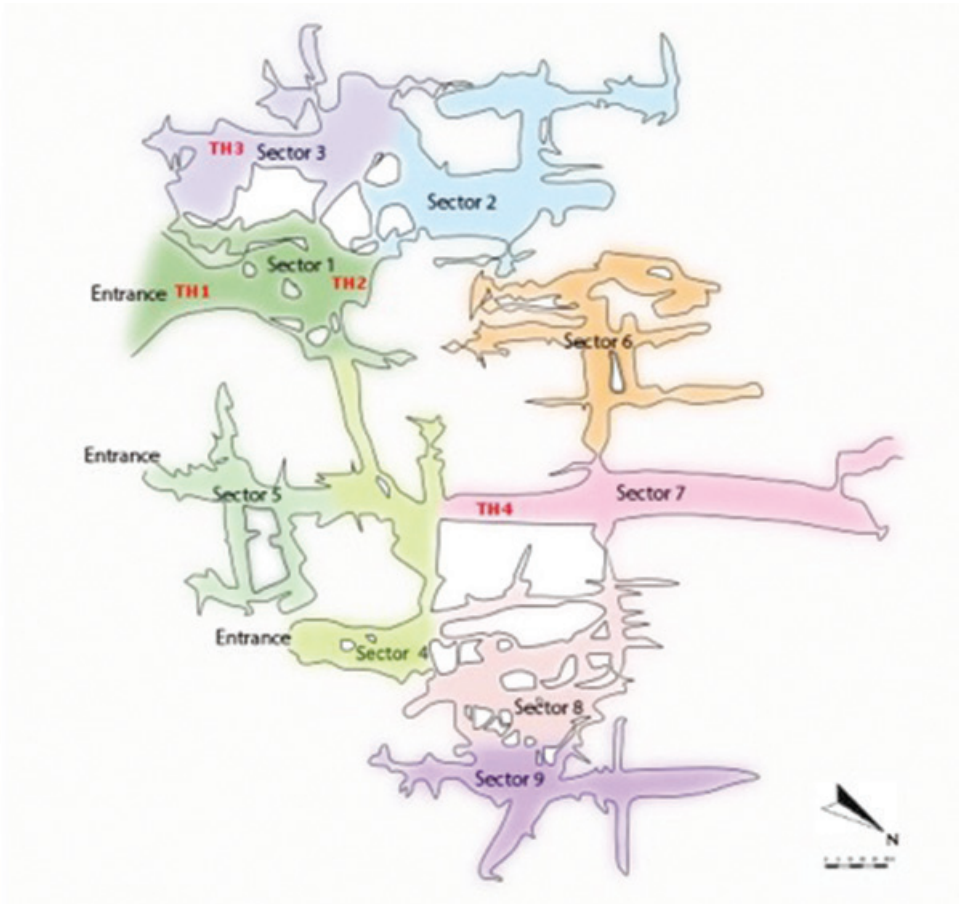

Figure 1. Lapa Nova Show cave map depicting the sectors: green tones correspond to entrance areas; the other six colors correspond to deeper regions of the cave. Legend: (TH) Temperature measurement areas.

Data analysis

In each sector we determined the richness, abundance and diversity of the invertebrate communities for each sampling period. The diversity calculation was made using the Shannon-Wiener index (Magurran 1988). In order to compare three entrance sectors with six inner sectors, we calculated mean richness and mean abundances values.

Accessing beta diversity components allow inferring about processes driving species distributions and biodiversity (Baselga and Orme 2012). Beta diversity components can result from species replacement (*turnover*) or species loss / gain (nestedness) (Baselga and Orme 2012). In order to determine temporal species composition differences, considering sectors between both sampling events, beta-partitioning diversity was calculated. For accessing temporal beta diversity, and also turnover and nestedness contribution for

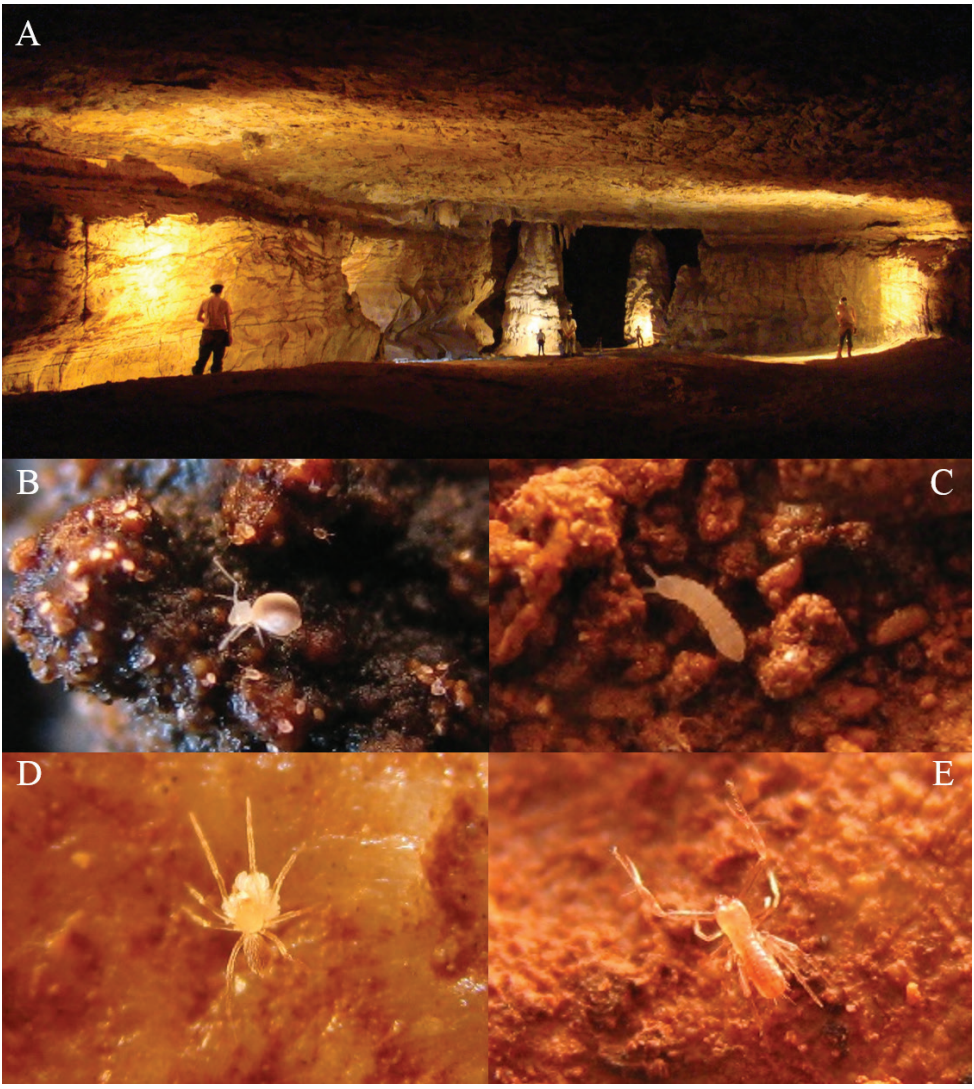

Figure 2. A Passage in Lapa Nova Cave **B, C, D, E** Examples of troglomorphic species recorded in the Northwest region of the state of Minas Gerais, Brazil. **B** Collembola: Arrhopalitidae: *Arrhopalites* sp.1 **C** Collembola: Poduromorpha, *Acherontides* sp.1 **D** Araneae: Ochyroceratidae sp.1 and **E** Pseudoscorpiones: Chthoniidae, *Pseudochthonius* sp.1.

total diversity, the BETA.TEMP function from the BETAPART package was used. According to Baselga and Orme (2012), this analysis compares two presence-absence species matrices from the sectors, at two different sampling events (April and September), and computes pairwise turnover components over time within each sampled sector. Total diversity was then computed by the Sorensen or Jaccard dissimilarity index. Finally, differences among beta diversity components, between entrances and inner sectors, were tested using ANOVA test, function AOV, from the STAT package. All analyses were

performed in R version 3.2.4 (R Development Core Team, 2014). In this work, we assumed that more stable sectors of the cave would present lower beta diversity values, reflecting lower species replacement or lost/gain rates, when compared to unstable sectors.

The similarity was evaluated among the fauna of all of the sectors of the cave, in the two periods. For that we used the Non-metric Multidimensional Scaling (n-MDS). The n-MDS was built based on the quantitative composition of the invertebrate fauna using the Bray-Curtis index. The existence of significant differences of the groups generated by the n-MDS was evaluated through ANOSIM, also done by the Bray-Curtis index. Finally, the SIMPER analysis was used to evaluate which species were responsible for such differences. All of the above analyses were conducted through the PRIMER 6.0 program.

Results

In Lapa Nova, a total of 24,482 individuals were recorded distributed in 187 species. From this total, 16,996 were registered in the April sampling, and 7,486 in the September sampling. The richness found in April was 160 species, while in September, that number was lower, 102 species. Of the total of 187 species, 85 were only observed in the first sampling event, 26 only in the second and 76 occurred in both periods. Diversity values were 2.79 and 2.87 for the first and second sampling events, respectively.

In April, Diptera was the order that presented the highest richness, with 33 species, followed by Araneae and Coleoptera with 31 and 29 species respectively. In September, the richness was much lower, the highest values being presented by Diptera and Araneae, both with 15 species, followed by Coleoptera with 12 species (Table 1).

Six troglomorphic species were found: *Arrhopalites* sp. (Collembola: Arrhopalitidae) (Fig. 2B), *Acherontides* sp. (Collembola: Hypogastruridae) (Fig. 2C), *Eukoenenia virgemdalapa* Souza and Ferreira, 2012 (Palpigradi: Eukoeneniidae), one Oonopidae (Araneae), one Chthoniidae (Pseudoscorpiones) (Fig. 2E) and one Styloniscidae (Isopoda) (Figure 2). Such troglomorphic species, did not present many differences in abundance and richness values among entrances and deeper sectors (Table 1).

The sectors of the cave did not present significant differences when comparing the two sampling events. There was a large overlap of species abundance in both sampling events by the similarity analysis conducted through n-MDS. Similarly, the ANOSIM test between the two periods was not significant ($p=0.062$) (Figure 3).

The average richness in entrance sectors and inner sectors was quite distinct. In entrance areas the average richness corresponded to 66 species in the first sampling event and 54 species in the second. Inner areas of the cave present an average of 28 species in April and 24 species in September (Table 2). The mean "*turnover*" of species was found to be 46.66 in entrance sectors, 44.72 in sectors at the mediations of entrance areas and 47.83 in inner sectors (Table 2). No pattern in differences in total beta diversity or in beta diversity components was found among inner or entrance sectors by the ANOVA test.

Table 1. Invertebrates collected and their abundance in the different sections in both seasons.

	SECTORS IN APRIL SAMPLING									SECTORS IN SEPTEMBER SAMPLING								
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**
NEMATODA																		
Nematoda sp.1		11		13					50									
OLIGOCHAETA																		
Lumbricidae sp.1	42						1			2					3			
HIRUDINEA																		
Hirudinida sp.1					1													
PULMONATA																		
Stylommatophora sp.1	1																	
Stylommatophora sp.2	2																	
Stylommatophora sp.3	3																	
Stylommatophora sp.4									1									
SARCOPTIFORMES																		
Acaridae sp.1	50																	
Astigmata sp.1										1								
Oribatida sp.1										1								
TROMBIDIFORMES																		
Anystidae (*Erythracarus* sp.1)	1			19			6		2	4	1		5	1		4	2	
Bdellidae (*Bdellodes* sp.1)	3					1							1		1		1	
Cheyletidae sp.1	1								6									
Rhagidiidae sp.1	5		1	9		1		14		4			2					
Teneriffiidae (*Neoteneriffiola xerophila*)				11									3					
IXODIDA																		
Ixodidae sp. 1	1																	
MESOSTIGMATA																		
Laelapidae sp. 1				1														
Laelapidae (*Stratiolaelaps* sp.1)				1			2		2	1								
Macronyssidae sp.						2	2					2						

	SECTORS IN APRIL SAMPLING									SECTORS IN SEPTEMBER SAMPLING								
Melicharidae (*Proctolaelaps* sp.1)		1				8									9			
Ologamasidae sp.1		2		1														
Podocinidae (*Podocinum* sp.1)									8									
ARANEAE																		
Araneae sp.1				1														
Araneae sp.2	1																	
Araneae sp.3					2													
Ctenidae (*Isoctenus* sp.1)	320	2	2	11	6	12	7	18	12	20	4	1	22	12	11	7	47	19
Deinopidae sp.1					1													
Gnaphosidae sp.1	1																	
Sicariidae (*Loxosceles variegata*)	71	8	8	180	72	34	57	855	932	115	3	12	144	49	29	139	995	917
Sicariidae (*Loxosceles* sp.2)			1															
Nemesidae sp.1								6										
Nemesidae sp.2	3																	
Caponiidae (*Nops* sp.1)								1	2	1			1				4	2
Oonopidae sp.1		4			1			3		4	4	10	2		1			
Pholcidae sp.1				2	5			1		2								
Salticidae sp.1										45								
Salticidae sp.1	11																	
Segestriidae sp.1					1													
Theridiidae sp.1		6																
Theridiidae sp.10	19	2	25	3	2	8	2			42	7	27	51	8	13	16		3
Theridiidae sp.11				2									9					
Theridiidae sp.12				1														
Theridiidae sp.13														2				
Theridiidae sp.14													1					
Theridiidae sp.2					1													
Theridiidae sp.3				1														
Theridiidae sp.4	1									1			3	5			1	
Theridiidae sp.5					1													

	SECTORS IN APRIL SAMPLING									SECTORS IN SEPTEMBER SAMPLING								
Theridiidae sp.6					1													
Theridiidae sp.7				2														
Theridiidae sp.8				1		1				1								
Theridiidae sp.9	1		11	1	1	4				18	3	11	22	4	5	7		
Theridiidae (*Theridium* sp.1)	10			11	18	7	3	1	21	2	1		1	10				
PALPIGRADI																		
Eukoeneniidae (*Eukoenenia* sp.1)	9							3		31		1					4	
Eukoeneniidae (*Eukoenenia virgemdalapa*)													1					
PSEUDOSCORPIONES																		
Cheiridiidae sp.1	20								2	2			4	6				
Chernetidae sp.1	26			5	3		15		4	7	1		2	1			1	30
Chernetidae sp.2					1					1								
Chthoniidae sp.1	3	3		2			1	1		2	2	6	4				1	
OPILIONES																		
Gonyleptidae sp.1	3																	
Gonyleptidae sp.2	1																	
Gonyleptidae sp.3	5	8	25		2	8	4	1		2	3	8	1		7	2	6	1
Opiliones sp.1 (juvenile)			3												1	1		
ISOPODA																		
Dubioniscidae sp.1					3													
Styloniscidae sp.1								1										
Plathyarthridae (*Trichorhina* sp.1)	10									4								
OSTRACODA																		
Ostracoda sp.1	21																	
Ostracoda sp.2										35								
CHILOPODA																		
Geophilidae sp.1					1													
Henicopidae sp.1	7									2								
Scutigeromorpha sp.1	1			1	11			1										

	SECTORS IN APRIL SAMPLING									SECTORS IN SEPTEMBER SAMPLING								
DIPLOPODA																		
Pseudonannolenidae (*Pseudonannolene* sp.1)	1			1				1	6					1				
COLLEMBOLA																		
Hypogastruridae (*Acherontides* sp.1)	1			10		3		5		1								
Arrhopalitidae (*Arrhopalites* sp.1)	4	2		3	1	2				1	4	1			1	1		
Collembola sp.1	19	12		4	1	2				14	1		4		2		13	
Collembola sp.2	184		1	40	12	14			2	130	8		35	4	16	2	117	
Collembola sp.3	2			3	23	36	31		3	13		1		1				
Collembola sp.4					3													
Collembola sp.5										19			1					
BLATTODEA																		
Blattodea sp.1	23	4	9	29	11	49	15	53	45	20	13	14	26	10	27	41	38	23
Blattodea sp.2		1																
COLEOPTERA																		
Carabidae sp.1	9									15			3					
Carabidae sp.2	16																	
Carabidae sp.3										1								
Cholevidae sp.1	266			6		85				21	3	3	1		3			
Elateridae sp.1									1									
Elateridae sp.2																		5
Histeridae sp.1										3							1	3
Carabidae (Larvae)	4																	
Coleoptera sp.10 (Larvae)							1											
Coleoptera sp.12 (Larvae)								1										
Coleoptera sp.8 (Larvae)						1												
Dermestidae sp.1 (Larvae)				1														
Dermestidae sp.2 (Larvae)				1														
Staphylinidae sp.2 (Larvae)	8																	
Staphylinidae sp.6 (Larvae)	200																	
Tenebrionidae sp.1 (Larvae)				1		1			1	1								

	SECTORS IN APRIL SAMPLING									SECTORS IN SEPTEMBER SAMPLING								
Tenebrionidae sp.2 (Larvae)					1			1		1			3	3		5	2	13
Ptiliidae sp.1										1								
Ptilodactylidae sp.1				4		1	2	2	11	1			1				3	
Staphylinidae sp.5	22					9												
Staphylinidae sp.1	7			1						2			1					
Staphylinidae sp.2					1													
Staphylinidae sp.3	5					2									3			
Staphylinidae sp.4	28					19				1					3			
Staphylinidae sp.6				1														1
Tenebrionidae (*Zophobas* sp.1)	1			8				10		2			4				1	
DIPTERA																		
Cecidomyiidae sp.1										1			1					
Conicera sp.1	5			2			7	3		52	61		47	9	16	2		
Diptera sp.1										7	11	3	3			3		
Díptera sp.2			1															
Díptera sp.4				1														
Díptera sp.5				1	2					1								
Drosophila sp.1	13			6	1	2	8		2003	7		1			1		1	13
Drosophilidae sp.2												1						
Chironomidae sp.1 (Larvae)	1									50								
Chironomidae sp.2 (Larvae)	19	10				1												
Chironomidae sp.3 (Larvae)	2	1								3			4					
Diptera sp.1 (Larvae)	61		100	3		16	25	2	2550			125			2			50
Diptera sp.2 (Larvae)	61		100	1		16	25	1	2250			125			3			50
Diptera sp.3 (Larvae)	8																	
Diptera sp.4 (Larvae)					1													
Diptera sp.5 (Larvae)					1													
Keroplatidae sp.1 (Larvae)	1	1																
Mycetophilidae sp.1 (Larvae)					1									2				
Milichiidae sp.1			16															

	SECTORS IN APRIL SAMPLING									SECTORS IN SEPTEMBER SAMPLING								
Muscidae sp.1											1							
Phoridae sp.2	1																	
Phoridae sp.1	2																	
Psychodidae (*Pericoma* sp.1)	2176	39		61	50		5	2	7	18	10	3	72		2	9		
Psycodidae sp.1	1																	
Psycodidae (*Lutzomyia* sp.1)						2		1	1						2	1		
Psycodidae sp.3						1			1				2			1		
Psycodidae sp.4		1																
Diptera sp.1 (Pupae)					1													
Ensifera																		
Phalangopsidae (*Eidmanacris* sp.1)	9			16	6			7		33	2		38	1			12	1
Phalangopsidae (*Endecous* sp.1)	87	47	14	43	10	14	31	149	124	26	17	42	20	10	16	16	125	31
Ensifera sp.1						1												
HEMIPTERA																		
Cicadellidae sp.1	2							1										
Cydnidae sp.1	1			1														
Cydnidae sp.2	2			1			2	1										
Cydnidae sp.3	17			1			19	4		5			1			1		
Emesinae sp.1	1			12	3	1	11	28	30	6			14	1		32	13	4
Hebridae sp.1	54			2						8			8					
Hebridae sp.2	10									14			2					
Hebridae sp.3	3																	
Hebridae sp.4	1																	
Hebridae sp.5	1																	
Homoptera sp.1													3					
Homoptera sp.1									8									
Reduviidae (*Zelurus* sp.1)	5									2								
Veliidae sp.1	8									1	1							
Hymenoptera																		
Braconidae sp.1	2									2								1

	SECTORS IN APRIL SAMPLING									SECTORS IN SEPTEMBER SAMPLING								
Hymenoptera sp.1									6									
Hymenoptera sp.3								1										
Formicidae (*Acromyrmex* sp.1)									1									
Formicidae sp.1				4				3					57					
Formicidae sp.2	1																	
Formicidae sp.3	1				2													
Formicidae sp.4				27	5			6		8							7	
ISOPTERA																		
Nasutitermitinae sp.1									3					1				1
LEPIDOPTERA																		
Arctiidae sp.1										1			1					
Noctuidae (*Hypena* sp.1)	21	3		98	9	12	82	75	1	131	19		187	35	10	39	13	
Lepidoptera sp.1 (Larvae)									1									
Tineidae sp.1 (Larvae)	5	10	38	6	40	17	104	22	342	58	1	3			3	19		
Tineidae sp.2 (Larvae)													1	1				
Erebidae (*Latebraria* sp.1)				1						2	1		4					
Lepidoptera sp.1 (Pupae)			1															
Tineidae sp.1	2			1			1			5		1	30	2	1	12	21	127
Tineidae sp.2	5	1	3		7	4	13	24	228	14	2	1	12	4		7	2	6
PSOCOPTERA																		
Ptiloneuridae (*Euplocania* sp.1)	6																	
Ptiloneuridae (*Euplocania* sp.2)	6																	
Lepidopsocidae (*Nepticulomima* sp.1)	8									3							5	
Psocoptera juvenile sp.4	3									5			4					
Psocoptera juvenile sp. 5	8									11			9					
Psyllipsocidae (*Psyllipsocus* sp.1)	8		14	9		5	21		15	81	7	49	64	5	31	117	29	117
Psyllipsocidae (*Psyllipsocus falcifer*)	14		24	15	2	7	35		24	137	11	83	109	8	52	198	48	198
Psyllipsocidae (*Psyllipsocus* sp.3)	1		2	1			3		3	14	1	8	11		5	20	5	20
Psyllipsocidae (*Psyllipsocus* sp.4)										3			13					

Table 2. Richness and Diversity values of the ten sectors in both sampling events.

Sector	April Richness	September Richness	Total Richness	Mean Richness	Turnover	Nestedness	β-Diversity
1 (entrance)	78	91	122	84.5	35.53	5.79	41.32
2 (inner)	29	23	38	26	39.13	7.02	46.15
3 (inner)	26	21	33	23.5	33.33	7.09	40.42
4 (entrance)	56	60	85	58	44.64	1.90	46.55
5 (entrance)	27	44	54	35.5	37.04	15.08	52.11
6 (inner)	30	38	47	34	30.00	8.23	38.24
7 (inner)	26	31	40	28.5	34.62	5.73	40.35
8 (inner)	29	37	50	33	44.83	6.69	51.52
9 (inner)	24	38	47	31	37.50	14.11	51.61

Table 3. SIMPER analysis. Species that mostly contributed to dissimilarity presented by entrance sectors in both sampling events.

Taxa	Individual Contribution	Cumulative %	September	April
Pericoma sp.1	24.59	24.59	30	762
Hypena sp.1	7.08	31.67	118	42.7
Loxosceles variegata	5.54	37.21	103	108
Psyllipsocus falcifer	5.34	42.55	84.7	10.3
Collembola sp.2	4.35	46.90	56.3	78.7
Isoctenus sp.1	3.64	50.54	18	112
Psyllipsocus sp.1	3.21	53.75	50	5.67
Cholevidae sp.1	3.02	56.77	7.33	90.7
Tineidae sp.1	2.62	59.39	19.3	17
Conicera sp.1	2.53	61.92	36	2.33
Theridiidae sp.10	2.17	64.08	33.7	8
Staphylinidae sp.6	1.95	66.04	0	66.7
Endecous sp.1	1.87	67.91	18.7	46.7
Eidimanacris sp.1	1.57	69.48	24	10.3

Entrance sectors and inner sectors also did not present significant differences among species composition and relative abundance between both sampling events (Figure 4), for the ANOSIM test through the Bray-Curtis index ($p > 0.40$). On the other hand, it was possible to observe a considerable variation in composition when observing each sector. The highest variation observed between the two sampling events was observed in sector one, at an entrance area, but also in sector nine, in a deep area of the cave.

The species that mostly contributed to the dissimilarity observed in the entrance sectors between both sampling events were: *Pericoma* sp. (Psychodidae, Diptera), responsible for approximately 24.5% of such differences; followed by *Hypena* sp, responsible for 7%; *Loxosceles variegata* (Sicariidae, Araneae), with 5.5%; *Psyllipsocus falcifer* (Psocoptera), with 5.3%; Collembola sp2, with 4.3% and *Isoctenus* sp (Ctenidae, Araneae) 3.6%

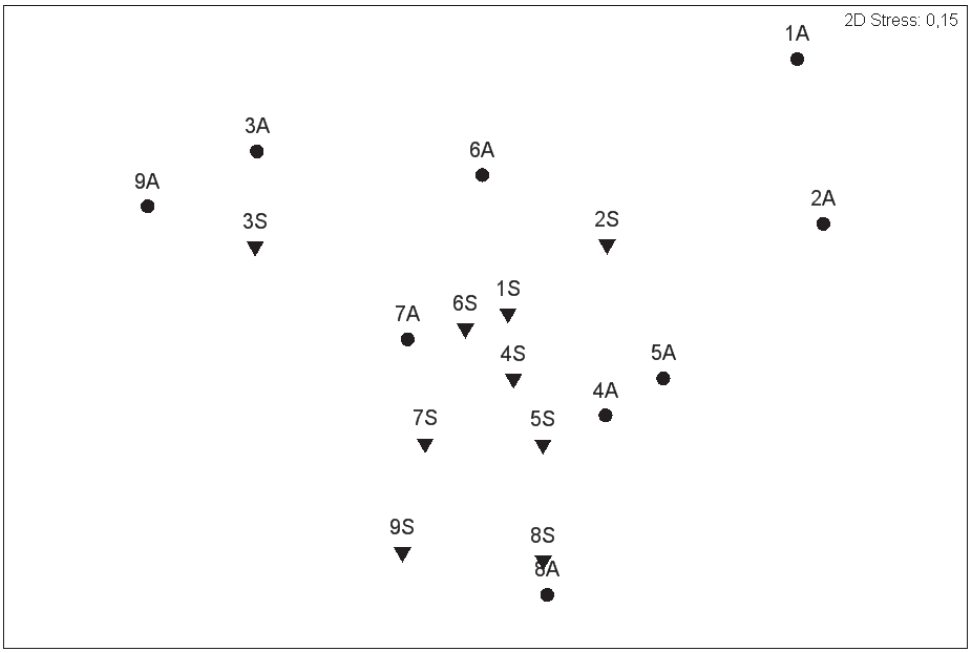

Figure 3. Multidimensional scaling (n-MDS) of sectors collected in April (circles) and September (triangles) periods, by the Bray-Curtis quantitative similarity index.

(Table 3). These species, together, accounted for 50% of the dissimilarities presented between the entrance sectors in both periods.

Deeper regions of the cave presented a different pattern. The species that most contributed to differences presented by such sectors were *Loxosceles variegata*, responsible for approximately 25.5% of such differences; followed by two species of dipteran larvae that together are responsible for 20% and *Psyllipsocus falcifer* (7.5%). Those species, together, were responsible for more than 50% of the similarities among the inner sectors in both periods (Table 4). None of the real cave species (troglobites) presented significant differences among sectors when considering both sampling events.

Discussion

The most common species found in Lapa Nova were those most ubiquitous in northwestern Minas Gerais limestone caves (Simões et al. 2014). The proportion of troglomorphic species in Lapa Nova (3.2% of the total) was above the average found for carbonatic caves present in the Brazilian Atlantic Forest (2.41%), and also for the proportion found in the Arcos-Pains-Doresópolis province, which is currently considered the area with the highest concentration of caves in Brazil (1.66%) (Zampaulo 2010, Souza-Silva et al. 2011). It worth pointing out that 72.34% of the caves sampled

Table 4. SIMPER analysis. Species that mostly contributed to dissimilarity presented by deep sectors in both sampling events.

Taxa	Individual Contribution	Cumulative %	September	April
Loxosceles variegata	25.56	25.56	349.17	315.67
Diptera sp.1	10.25	35.81	29.50	448.83
Diptera sp.2	9.55	45.35	29.67	398.67
Psyllipsocus falcifer	7.59	52.94	98.33	15.00
Drosophila sp.1	4.94	57.88	2.67	335.50
Psyllipsocus sp.1	4.48	62.36	58.33	9.17
Tineidae sp.1 (Larvae)	4.21	66.57	4.33	88.83
Endecous sp.1	3.51	70.08	41.17	63.17

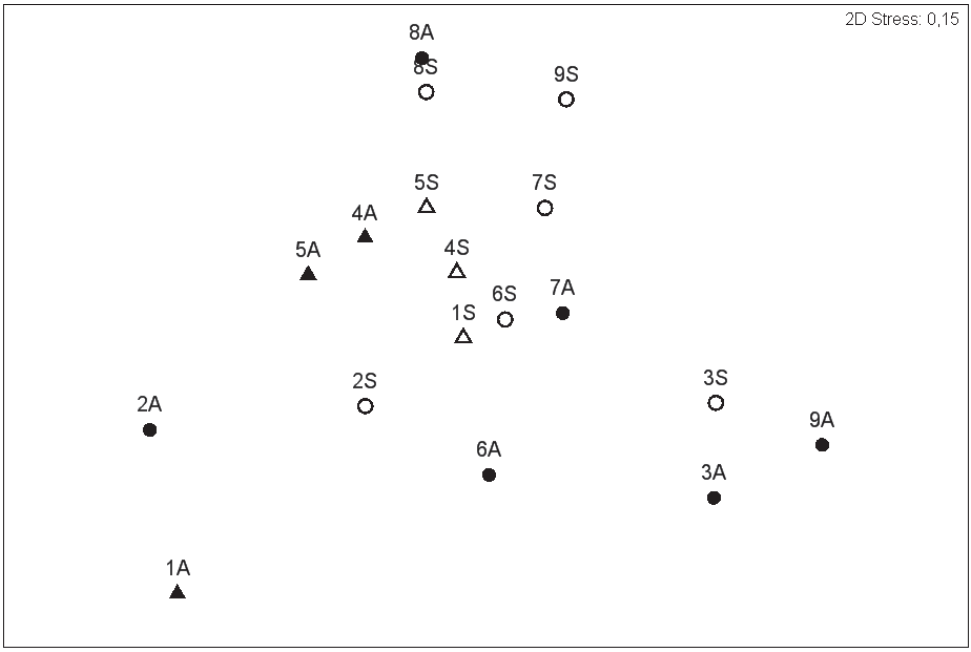

Figure 4. Multidimensional scaling (n-MDS) of sectors in entrance areas in April (A-black triangles), and sectors in deep areas of the cave in April (A-black circles), and sectors in entrance areas in September (S-white triangles), and sectors in deep areas of the cave in September (S-white circles), by the Bray-Curtis quantitative similarity index.

in northwestern Minas Gerais present only one or no troglobitic species, while only 4.26% of the caves possess more than six troglobitic species (Simões et al. 2014). Accordingly, in a regional context, Lapa Nova Cave can be considered as an extremely important cave regarding endemic cavernicolous species with 6 species, being considered as a priority cave for conservation in the region (Simões et al. 2014).

Temporal differences in species composition

Despite the common statement that caves are stable environments, such environments can have strong responses to changing surface climate (Tobin et al. 2013), and also to changes inside caves (Rocha 2013). Studies concerning cave community variations related to the sampling period concentrate on temperate areas, where those variations are more striking, with an important impact on the fauna (Romero 1983, 2002, 2009, Tobin et al. 2013). In Brazilian caves, there is an indication of a less pronounced variation, though it is, in many caves, quite noticeable (Gomes et al. 2000). While some populations underwent more drastic alterations, others remained with their abundance and spatial distribution little changed (Gomes et al. 2000, Ferreira et al. 2015). The same was observed at Lapa Nova Cave, where some species had a severe reduction in the number of specimens visible in the cave, while others showed an increase in abundance. Some species presented only small abundance alterations, as in the case of the brown spider (*Loxosceles variegata* Simon, 1897). It is important to mention that this is a troglophilic species, which is prone to tolerate higher environmental variations, especially when compared to troglobitic species (Trajano and Bchuette 2010). However, such species showed variation in individual distribution along cave sectors. This dispersion pattern presented by brown spiders among cave sectors was already demonstrated in Lavoura Cave (Minas Gerais state, Brazil), in which some specimens migrated more than 80 meters during a month (Ferreira et al. 2005). The authors attributed such movement to an irregular food resource distribution within the cave. Therefore, spiders had to travel longer distances searching for prey.

Considering other biological parameters such as richness and diversity: richness usually increases in the rainy period; diversity, in turn, does not show a well-established pattern (Ferreira 2004). At Lapa Nova Cave, richness and abundance increased in April, while diversity was higher in September. Richness and abundance variation between two sampled periods can also reflect reproductive periods of some species (Pellegatti-Franco 2004). Moreover, studies indicate that the morphology of the cave walls and the climatic conditions regulate the distribution of moth species within a cave ecosystem (Bourne 1976). Furthermore, the reduction of percolation water availability should favor a wider dispersion of the organisms throughout the cave, in search of more favorable microclimatic conditions. During the September field sampling, the drier sampling event, it was observed *in loco* that organisms were concentrated in areas where there were patches of moisture on the soil, like small pools on areas. These areas were distributed in scattered points in the cave, thus favoring a wider dispersion of the organisms.

Spatial distribution patterns

Cave and subterranean mine entrance areas present a greater density of many populations (Culver and Poulson 1970, Peck 1976), and also higher richness, especially when

compared with inner portions of the caves (Prous et al. 2004, 2015). The same was observed at Lapa Nova Cave, where the entrance areas presented a richness average higher than those observed in the inner areas of the cave. Considering cave entrances as ecotones, the high richness in such transitional zone communities is certainly expected, since these areas present species from both adjacent environments plus exclusive species (Prous et al. 2015).

Beyond the higher richness in entrance areas, one would expect less variation to be found in the inner communities when compared to those located near the entrances, due to higher environmental stability. However, at the Lapa Nova cave such tendency was not observed. Despite the higher climatic differences at entrances areas, inner communities did not vary less, in composition, than those located near entrances, as observed though temporal beta diversity. The high species turnover in all sampled sectors reflects species replacements (Baselga and Orme 2012), due to cave community instabilities. This "paradox" observed in Lapa Nova Cave is probably related to a singular condition of this cave. This cave experiences intense tourism, concentrated over a single period of the year, during a religious festival in honor of the Virgem da Lapa ("Virgin of the Cave"), which occurs early in the month of May. This intense tourism has severe impacts on invertebrate fauna, as well as changes in the spatial distributions of the community (Pellegrini and Ferreira 2012). Tourism is more intense at the main entrance of the cave. This area corresponds to Sector 1, that is one of the most variables sectors of the cave considering the similarity between the two periods analyzed. Therefore, the fauna moves to inner portions of the cave (Pellegrini and Ferreira 2012), generating a huge population displacement into the entire cavity. Tourism also influences microbial distribution. It causes conidial transfer from different sectors in the cave or even allochtonous import from the epigean system (Taylor et al. 2013). In this sense, tourism causes instability in all sectors of the cave, since it is not restricted to entrance areas.

Tourism instabilities

The touristic use of a cave, if uncontrolled, can potentially lead to population size reduction of some species, and this is especially dramatic considering troglobitic species. At Areias Cave System in Brazil, intense tourism led to a strong reduction in the population size of the cavefish, *Pimelodella kronei* (Guil and Trajano 2013). The same observation occurred in a study conducted at Ursilor Cave (Romania) before and after it's opening as a show cave (Moldovan et al. 2003). On the other hand, other studies have shown obligate cave species (majority represented by arachnids, crustaceans and beetles) co-existing with cave tourism, as observed in many show caves around the world, such as the Lapa Nova Cave in Brazil, Mammoth Cave, Kentucky, System Postojna-Planina and Krizna Jama Cave in Slovenia, Vjetrenica Jama Cave in Bosnia-Herzegovina, La Verna chamber, in France, among others (Pellegrini and Ferreira 2012, Culver and Sket 2002, Faille et al. 2014). Unfortunately, for most show caves around

the world, the assessment of cave fauna has only occurred after the establishment of the tourism, so the pristine conditions of those caves, in most cases, remains unknown.

It worth stating that at La Verna chamber (France), cave tourism did not impose any negative impact on the troglobitic species, which include 18 endemic hypogean invertebrates (Faille et al. 2014). However, tourism at La Verna chamber has some particularities that allow such non-impact: 1) the huge chamber of the cave that prevents significant microclimatic changes, and also offers suitable micro-habitats for invertebrates to shelter themselves from disturbance and illumination; 2) Cave tourism occurs in limited duration during the year and limited spatial coverage, in accordance with cave size (Faille et al. 2014). On the other hand, in Lapa Nova Cave, touristic passages occur regardless of cave conduit size and species distribution, leading to severe disturbances to the cave fauna. At Vjetrenica Jama, although 60 obligate subterranean species co-exist with tourism, some stygobiont species have disappeared from cave areas because of leakage from batteries left in the cave by visitors (Culver and Sket 2002).

Conclusions

Lapa Nova cave revealed to be a quite peculiar system, not only regarding its dimensions and geology, but also as to the patterns existent in the associated fauna. Visitation at the cave probably altered the expected natural community distribution pattern, as has already been stated in Romania show caves studies (Moldovan et al. 2013), imposing a new organization of the communities, driven by the unstable conditions imposed by cave tourism. The need to conduct studies such as this in show caves is evident, although with longer temporal scales so that one can confirm the seasonal changes undergone by the cave communities. Furthermore, studies regarding sampling events before tourism establishment are especially important in order to evaluate population sizes, distributions and conditions to guarantee sustainable cave use.

Acknowledgments

To colleagues Érika Linzi Taylor, Maysa Fernanda de Souza and Marconi Souza Silva, for their help with field work. We also would like to thank Mr. Severino, that for many years ensured the protection of the Lapa Nova cave. To the researchers, for the identification of taxonomic groups: Thaís Oliveira do Carmo (Psocoptera), Leopoldo Bernardi (Acari), Maysa Fernanda de Souza (Palpigradi) and Daniele Pompeu (Pseudoscorpiones). To Professor Paulo Pompeu, for his assistance with statistical analyses. To Professor Júlio Louzada and Rogério Parentoni Martins, for their suggestions. To Votorantim Metais and Carste Consultores Associados Ltda for the financial support given to this work. This work was supported by the FAPEMIG under Grant [no. APQ-01854-09]; Conselho Nacional de Pesquisa (CNPq) provided funding to R.L.F. under Grant [CNPq grant nr. 304682/2014-4].

References

Auler AS, Rubbioli E, Brandi R (2001) As grandes cavernas do Brasil, Belo Horizonte: Grupo Bambuí de Pesquisas Espeleológicas, 228 pp.

Auler AS, Moura VMA, David H, Ferreira RL, Taylor ELS, Pellegrini TG, Hubbe A (2009) Plano de Manejo Espeleológico – Lapa Nova, Vazante, MG. Apresentado à Votorantim Metais, 118 pp.

Baselga A, Orme CDL (2012) Betapart: an R package for the study of beta diversity. Methods in Ecology and Evolution 3(5): 808–812. doi: 10.1111/j.2041-210X.2012.00224.x

Bernard LFO, Souza-Silva M, Ferreira RL (2010) Efeitos do uso turístico sobre cavidades subterrâneas artificiais: subsídeos para o uso antrópico de sistemas subterrâneos. Tourism and Karst Areas 4(2): 71–88.

Bourne JD (1976) Notes préliminaires sur la distribution spatiale de *Meta menardi*, *Triphosa dubitata*, *Triphosa sabaudiata*, *Nelima aurantiaca* et *Culex pipiens* au sein dùn écosystème cavernicole (Grotte de la Scierie: Hte.-Savoie). International Journal of Speleology 8: 253–267. doi: 10.5038/1827-806X.8.3.2

Culver DC, Poulson TL (1970) Community boundaries: faunal diversity around a cave entrance. In Annales Spéléologie 25(4): 853–860.

Culver DC, Sket B (2002) Biological Monitoring in Caves. Acta Carsologica 31(1): 55–64.

Faille A, Bourdeau C, Deharveng L (2015) Weak impact tourism activities on biodiversity in a subterranean hotspot of endemism and its implications for the conservation of cave fauna. Insects Conservation and Diversity 8: 205–215. doi: 10.1111/icad.12097

Ferreira RL (2004) A medida da complexidade ecológica e suas aplicações na conservação e manejo de ecossistemas subterrâneos. PhD Thesis, Universidade Federal de Minas Gerais, Belo Horizonte, 158 pp.

Ferreira RL, Prous X, Machado SF, Martins RP (2005) Population dynamics of *Loxosceles similis* (Moenkhaus, 1898) in a Brazilian dry cave: a new method for evaluation of population size. Revista Brasileira de Zoociências 7(1): 129–141.

Ferreira RL, Martins VM, Paixão EA, Souza-Silva M (2015) Spatial and temporal fluctuations of the abundance of Neotropical cave-dwelling moth *Hypena* sp. (Noctuidae, Lepidoptera) influenced by temperature and humidity. Subterranean Biology 16: 47–60. Doi: 10.3897/subtbiol.16.5137

Gomes FTMC, Ferreira RL, Jacobi CM (2000) Comunidade de artrópodes de uma caverna calcária em área de mineração: composição e estrutura. Revista Brasileira de Zoociências 2(1): 77–96.

Guil ALR, Trajano E (2013) Dinâmica populacional do bagre cego de Iporanga, *Pimelodella kronei*: 70 anos de estudos. Revista da Biologia 10(2): 34–39. doi: 10.7594/revbio.10.02.06

Huppop K (2005) Adaptation to Low Food. In: Culver DC, White WB (Eds) Encyclopedia of caves, California, Elsevier Academic Press, 4–10.

Lobo HAS, Perinotto JAJ, Poudou S (2009) Análise de agrupamentos aplicada à variabilidade térmica da atmosfera subterrânea: contribuição ao zoneamento ambiental microclimático de cavernas. Revista de estudos ambientais 11(1): 22–35.

Lobo HAS, Boggiani PC, Perinotto JAJ (2015) Speleoclimate dynamics in Santana Cave (Petar, São Paulo State, Brazil): general characterization and implications for tourist management. International Journal of Speleology 44(1): 61–73. doi: 10.5038/1827-806X.44.1.6

Magurran A (1988) Ecological Diversity and Its Measurement. British Library, Cambridge, 177 pp. doi: 10.1007/978-94-015-7358-0

Moldovan OT, Racovitza G, Rajka G (2003) The impact of tourism in Romania show caves: the example of the beetle populations in the Ursilor Cave of Chiscau (Transilvania, Romania). Subterranean Biology 1: 73–78.

Moseley M (2009) Are all caves ecotones? Cave and Karst Science 36(2): 53–58.

Novak T, Perc M, Lipovsek S, Janzekovic F (2012) Duality of terrestrial subterranean fauna. International Journal of Speleology 41(2): 57–64. doi: 10.5038/1827-806x.41.2.5

Oliveira MA, Ferreira RL, Carneiro MA, Diotaiuti L (2008) Ecology of Cavernicola pilosa barber, 1937 (Hemiptera: Reduviidae: Triatominae) in the Boa Esperança Cave, Tocantins, Brazil. Ecotropica 14: 63–68.

Peck SB (1976) The Effect of cave entrances on the distribution of cave-inhabiting terrestrial arthropods. International Journal of Speleology 8: 309–321. doi: 10.5038/1827-806X.8.4.1

Pellegatti-Franco F (2004) Biologia e ecologia populacional de *Ctenus fasciatus* Mello-Leitão e Enoploctenus cyclothorax (Bertkau) em cavernas do Alto Ribeira, Iporanga, São Paulo (Araneae: Ctenidae). PhD Thesis, Universidade de São Paulo, São Paulo, 136 pp.

Pellegrini TG, Ferreira RL (2012) Management in a neotropical show cave: planning for invertebrates conservation. International Journal of Speleology 41(2): 361–368. doi: 10.5038/1827-806X.41.2.19

Prous X, Ferreira RL, Martins RP (2004) Ecotone delimitation: epigean-hypogean transition in cave ecoystems. Austral Ecology 29: 374–382. doi: 10.1111/j.1442-9993.2004.01373.x

Prous X, Ferreira RL, Jacobi CM (2015) The entrance as a complex ecotone in a Neotropical cave. International Journal of Speleology 44(2) 177–189. doi: 10.5038/1827-806X.44.2.7

R Development Core Team (2014) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna. http://www.R-project.org/

Rocha PA (2013) Quiropterofauna cavernícola: composição, estrutura de comunidades, distribuição geográfica e aspectos ecológicos das populações. PhD Thesis, Universidade Federal da Paraíba-João Pessoa, 164 pp.

Romero A (2009) Cave Biology. Cambridge University Press, New York, 319 pp. doi: 10.1017/CBO9780511596841

Romero A (2002) The life and work of a little known biospeleologist: Theodor Tellkampf. Journal of Spelean History 36(2): 68–76.

Romero A (1983) Introgressive hybridization in a population of Astyanax fasciatus (Pisces: Characidae) at La Cueva Chica. National Speleological Society Bulletin 45: 81–5.

Simões MH, Souza-Silva M, Ferreira RL (2014) Cave Invertebrates in Northwestern Minas Gerais State, Brazil: Endemism, Threats and Conservation Priorities. Acta Carsologica 43(1): 159–174. doi: 10.3986/ac.v43i1.577

Simões MH, Souza-Silva M, Ferreira RL (2015) Cave physical attributes influencing the structure of terrestrial invertebrate communities in Neotropics. Subterranean Biology 16: 103–121. doi: 10.3897/subtbiol.16.5470

Souza-Silva M, Martins RP, Ferreira RL (2011) Cave lithology determining the structure of the invertebrate communities in the Brazilian Atlantic Rain Forest. Biodiversity and Conservation 20: 1713–1729. doi: 10.1007/s10531-011-0057-5

Souza Silva M, Ferreira RL (2016) The first two hotspots of subterranean biodiversity in South America. Subterranean Biology 19: 1–21. doi: 10.3897/subtbiol.19.8207

Taylor EL, Stoianoff MAR, Ferreira RL (2013) Mycological study for a management plan of a neotropical show cave (Brazil). International Journal of Speleology 42(3): 267–277. doi: 10.5038/1827-806X.42.3.10

Tobin BW, Hutchins BT, Schwartz BF (2013) Spatial and temporal changes in invertebrate assemblage structure from the entrance to deep-cave zone of a temperate marble cave. International Journal of Speleology 42(3): 203–214. doi: 10.5038/1827-806X.42.3.4

Trajano E, Bichuette ME (2010) Diversity of Brazilian subterranean invertebrates, with a list of troglomorphic taxa. Subteranean Biology 7: 1–16.

Zampaulo RA (2010) Diversidade de invertebrados cavernícolas na província espeleológica de Arcos, Paíns e Doresópolis (MG): Subsídios para a determinação de áreas prioritárias para a conservação. MD Thesis, Universidade Federal de Lavras, Lavras, Brazil, 207 pp.

Subterranean Biology 20: 39–50 (2016)
doi: 10.3897/subtbiol.20.9693
http://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

First definitive record of a stygobiotic fish (Percopsiformes, Amblyopsidae, *Typhlichthys*) from the Appalachians karst region in the eastern United States

Matthew L. Niemiller[1], Kirk S. Zigler[2], Pamela B. Hart[3], Bernard R. Kuhajda[4], Jonathan W. Armbruster[3], Breanne N. Ayala[2], Annette S. Engel[5]

1 *Illinois Natural History Survey, University of Illinois Urbana-Champaign, Champaign, IL 61820* **2** *Department of Biology, University of the South, Sewanee, TN 37383* **3** *Department of Biological Sciences, Auburn University, Auburn, AL 36849* **4** *Tennessee Aquarium Conservation Institute, Chattanooga, TN 37402* **5** *Department of Earth and Planetary Sciences, University of Tennessee, Knoxville, TN 37996*

Corresponding author: *Matthew L. Niemiller* (cavemander17@gmail.com)

Academic editor: *O. Moldovan* | Received 29 June 2016 | Accepted 14 September 2016 | Published 8 November 2016

http://zoobank.org/5A60148E-4803-42AB-B85F-4F05581D3643

Citation: Niemiller ML, Zigler KS, Hart PB, Kuhajda BR, Armbruster JW, Ayala BN, Engel AS (2016) First definitive record of a stygobiotic fish (Percopsiformes, Amblyopsidae, *Typhlichthys*) from the Appalachians karst region in the eastern United States. Subterranean Biology 20: 39–50. doi: 10.3897/subtbiol.20.9693

Abstract

In the central and eastern United States, cavefishes have been known historically only from the Interior Low Plateau and Ozarks karst regions. Previously, cavefishes were unknown from the Appalachians karst region, which extends from southeastern New York southwestward into eastern Tennessee, northwestern Georgia, and northeastern Alabama. Here we report the discovery of a new population of the amblyopsid cavefish *Typhlichthys subterraneus* Girard, 1859 from a cave in Catoosa County, Georgia, that significantly extends the known distribution of the species. The cave is located in the Appalachian Valley and Ridge physiographic province and Appalachians karst region, and represents the first definitive report of a stygobiotic fish from the Appalachians karst region. Genetic analyses of one mitochondrial and one nuclear locus from the cavefish indicate this population is closely allied with populations that occur along the western margins of Lookout and Fox mountains in Dade County, Georgia, and populations to the northwest in southern Marion County, Tennessee. It is likely that these populations are also related to those from Wills Valley, DeKalb County, Alabama. The distribution of this new population of *T. subterraneus* and its close allies pre-dates the emergence of a Tennessee-Coosa River drainage divide in the Pliocene. The potential exists to discover additional populations in caves within the Appalachians karst region in Catoosa County and northward into Hamilton County, Tennessee.

Keywords
Appalachian Valley and Ridge, Catoosa County, cavefish, Cumberland Plateau, Georgia, range extension

Introduction

Of the more than 50,000 caves reported in the United States, about 30% occur in the states of Tennessee, Alabama, and Georgia (TAG). The two most biodiverse karst regions in the United States – the Interior Low Plateau (ILP) and Appalachians – occur in this region (Culver et al. 2000, Culver and Pipan 2009). The ILP is comprised of horizontal strata of Ordovician through Mississippian age that extend from southern Illinois and Indiana, southward through Tennessee and Kentucky and into northern Alabama. The escarpments of the Cumberland Plateau in Kentucky, Tennessee, Alabama, and Georgia are included in the ILP karst region (Culver et al. 2000). The ILP and Appalachians karst regions are proximal to each other near the junction of TAG state borders, although the boundary between the ILP region and the Appalachians karst region, and Appalachian Valley and Ridge (AVR) physiographic province, is somewhat arbitrary. Caves in the Appalachians karst are predominantly developed within Paleozoic rocks of an ancient fold-and-thrust belt associated with compression during Alleghenian orogenesis of the Appalachian Mountains (Hatcher et al. 2007, Hatcher 2010). The AVR physiographic province is comprised of parallel ridges of sandstones with intervening structural valleys of folded and faulted shales and carbonates that extend from southeastern New York to eastern Tennessee, northwestern Georgia, and northeastern Alabama between the Blue Ridge Mountains to the east and the Appalachian Plateau (specifically, the Cumberland Plateau) to the west.

The ILP and Appalachian karst regions contain the most caves and have the greatest richness of troglobiotic taxa in the United States (Culver et al. 2003, Hobbs 2012). In particular, a hotspot of subterranean biodiversity and endemism has been identified near the contact of the ILP and Appalachians karst regions along the escarpments of the Cumberland Plateau in northeastern Alabama and south-central Tennessee (Culver et al. 1999, 2000, 2006, Christman et al. 2005, Niemiller and Zigler 2013). Species richness in the Appalachians karst region (and AVR) is less than half that observed in the ILP in the TAG region, and AVR subterranean fauna are distinct from ILP fauna. Only 9% of the 200+ troglobionts in Tennessee occur in both karst regions (Niemiller and Zigler 2013).

Several factors may explain differences in species richness between these the ILP and Appalachians karst regions, such as differences in habitat availability, habitat connectivity, historical factors, and surface productivity (Christman and Culver 2001, Culver et al. 2006, Niemiller and Zigler 2013). Cave density has been viewed as a surrogate for habitat availability and connectivity because it positively correlates with regional species richness (Christman and Culver 2001, Culver et al. 2003, 2006). Cave density is considerably lower in the southern Appalachians karst region compared to the ILP in the TAG region. Moreover, the folded and faulted cave-bearing strata in the Appalachians karst region are dissected and discontinuous compared to horizontal

Figure 1. *Typhlichthys subterraenus* collected 25 November 2015 from Crane Cave (GCZ80), Catoosa County, Georgia. Photograph by B.R. Kuhajda.

strata of the ILP. A major zone of faulting along the eastern escarpment of the Cumberland Plateau in the Appalachians karst region has been hypothesized to act as a stratigraphic barrier to subterranean dispersal between the two karst regions (Barr and Holsinger 1985, Miller and Niemiller 2008, Niemiller et al. 2008, 2009), which may explain why so few species occur in both karst regions.

Typhlichthys subterraneus s.l. Girard, 1859 is one of the most wide-ranging cavefishes in the world (Proudlove 2006, Niemiller and Poulson 2010). In the TAG region, this cavefish is known from >180 caves in the ILP, with the greatest concentration of occurrences in central Tennessee and northern Alabama (Niemiller et al. 2013b,c). In Georgia, *T. subterraneus* is known only from four caves developed in Mississippian Bangor Limestone along the western margins of Lookout Mountain and Fox Mountain in Dade County, Georgia (Cooper and Iles 1971, Freeman and Niemiller 2009, Niemiller et al. 2012a, 2013b,c). Here we report the discovery of a population of the Southern Cavefish (*Typhlichthys subterraneus*) from Crane Cave in Catoosa County, northwestern Georgia (Fig. 1), located in the center of the AVR physiographic province and the Appalachians karst region. Not only does this record represent a significant range extension for this species, but it also represents the first definitive report of a stygobiotic fish from the Appalachians karst region.

Materials and methods

Study site

Crane Cave (Georgia Speleological Survey cave no. GCZ80) is located ca. 7 km SSE of Fort Oglethorpe, Georgia, in the South Chickamauga Creek watershed. Crane Cave formed in the Ordovician Newala Limestone, and has 292 m of mapped length with 11 m of vertical extent and three entrances. A small stream runs through the cave and emerges at the spring entrance. The stream begins in a large pool at the back of the

cave called "The Found Sea." The pool is ca.10 m in length and ca. 6 m in width, and has a mud/silt substrate bottom. The full extent of the pool is unknown, as it extends underneath a ledge at the back of the cave. At base level, water depth is ca. 2 m deep in the deepest portion of the pool.

Cavefish survey

Crane Cave was visited on four occasions: 10 August 2015, 18 August 2015, 29 October 2015, and 25 November 2015. The Found Sea and other aquatic habitats were sampled using time-constrained visual surveys with headlamps and handheld dive lights. Richness and abundance data for aquatic fauna were recorded, and a concerted effort was made to capture fish with handheld dipnets. A voucher specimen and tissue sample (fin clip) was obtained for morphological and genetic analyses.

Molecular methods and analyses

Genomic DNA was extracted from fin clips using the EZNA DNA Extraction Kit (Omega Biotek). Two gene loci were chosen from six previously used by Niemiller et al. (2012b) to determine the genetic identify and relationships of the Crane Cave population to other *Typhlichthys* populations. The protein-coding mitochondrial NADH dehydrogenase 2 (ND2) gene was amplified by PCR with primers TyCon1F (5'-TGAACCCTTTCATCCTAATAGCC-3') and TyCon1R (5'-GGTTGTGAGGAGGGTCAGG-3'). Each PCR reaction contained 8.5 µL of purified water, 12.5 µL Master Mix (Promega Corporation), 2.0 µL DNA template, 1.0 µL each of 10 µM forward and reverse primers. Amplification began with an initial denaturation of 94 °C for 30 seconds, followed by 30 cycles of 94 °C denaturing for 30 seconds, annealing at 51.2 °C for 30 seconds, elongation at 72 °C for 75 seconds, then a final elongation step of 10 minutes. The gene sequence was 957 base pairs (bp) long. A 774-bp section of the first intron of the ribosomal nuclear encoded S7 gene was amplified with the primers S7Con1F (5'-TCTGCAGGATGGAAGATTTTGT-3') and S7Con1R (5'-GCTTGTACTGAACATGGCCC-3'). The PCR reactions contained the same amount and concentration of reagents as the ND2 reaction. The initial denaturation for amplification began at 95 °C for 60 seconds, followed by 30 cycles of denaturation at 95 °C for 30 seconds, annealing at 60 for 60 seconds, elongation at 72 °C for 2 minutes, followed by final elongation at 72 °C for 10 minutes. PCR products were cleaned using ExoSAP-IT (Affymetrix) and bidirectionally sequenced at Genewiz, Inc. (Cambridge, Massachusetts, USA). Unique sequences generated for the Crane Cave sample were accessioned into GenBank (ND2: KX173801 and S7: KX246929).

Forward and reverse sequences were aligned into contigs and edited with manual verification using Geneious v. 6.0.6 (Biomatters Ltd.). Maximum likelihood gene

trees were generated for both ND2 and S7 loci with raxmlGUI v.1.31 (Silvestro and Michalak 2012). Codon partitioning according to Niemiller et al. (2012b) was utilized for ND2. For both loci, a maximum likelihood + thorough bootstrap analysis was conducted with 10 replicates of 100 runs utilizing the cavefishes *Speoplatyrhinus poulsoni* Cooper & Kuehne, 1974 and *Amblyopsis spelaea* DeKay, 1842 as outgroup taxa.

Results

A single cavefish was observed in The Found Sea of Crane Cave but evaded capture during an initial bioinventory on 10 August 2015. No cavefish were observed during two subsequent trips on 18 August 2015 and 29 October 2015. Two cavefishes were observed on 25 November 2015. One specimen was collected and retained as a voucher specimen (Fig. 1). The specimen was identified as *Typhlichthys subterraneus* by the lack of external eyes (vs. presence in *Chologaster* and *Forbesichthys*), presence of one row of exposed neuromasts on each half of the caudal fin (*Amblyopsis, Speoplatyrhinus*, and *Troglichthys* have four to six rows, two to three on each half of the caudal fin), the presence of branched rays in the pectoral fins (vs. unbranched in *Speoplatyrhinus*), the lack of pelvic fins (vs. presence in *Amblyopsis*), and nine dorsal-fin rays (vs. 7–8 in *Troglichthys*). In addition, only *Typhlichthys*, among stygobiotic amblyopisids, is known to have an extensive pigment response when exposed to light (Eigenmann 1909; Poulson 1963). The Crane Cave specimen has extensive melanophore development particularly along the edges of myomeres, on the head, and at the bases of the median fins (*Amblyopsis, Speoplatyrhinus,* and *Troglichthys* have far fewer melanophores with less melanin, and color is not generally noticeable in preserved specimens). The specimen was cataloged into the Auburn University Museum of Natural History (AUM 67212) and a tissue sample (fin clip) was accessed into the Auburn University Fish Tissue Collection (AUFT 2651).

Other notable fauna observed during the four biological surveys at Crane Cave included aquatic species *Crangonyx antennatus* Cope & Packard, 1881 (Amphipoda: Crangonyctidae), *Caecidotea richardsonae* Hay, 1901 (Isopoda: Asellidae), and *Cottus* sp. (Scorpaeniformes: Cottidae), and terrestrial species *Hesperochernes mirabilis* (Banks, 1895) (Pseudoscorpiones: Chernetidae), *Bishopella* sp. (Opiliones: Phalangodidae), *Amoebaleria* sp. (Diptera: Heleomyzidae), and *Eidmanella pallida* (Emerton, 1875) (Araneae: Nesticidae).

Molecular results indicated that the Crane Cave specimen was most closely related to the *T. subterraneus* populations designated lineage A in both the ND2 and S7 phylogenies (Niemiller et al. 2012b). In the ND2 phylogeny (Fig. 2), the Crane Cave specimen was sister to a clade containing populations from Long's Rock Wall (GDD101) and Limestone Caverns (GDD140) from Dade County, Georgia, in the Lookout Creek watershed, and the closest populations in geographical proximity to

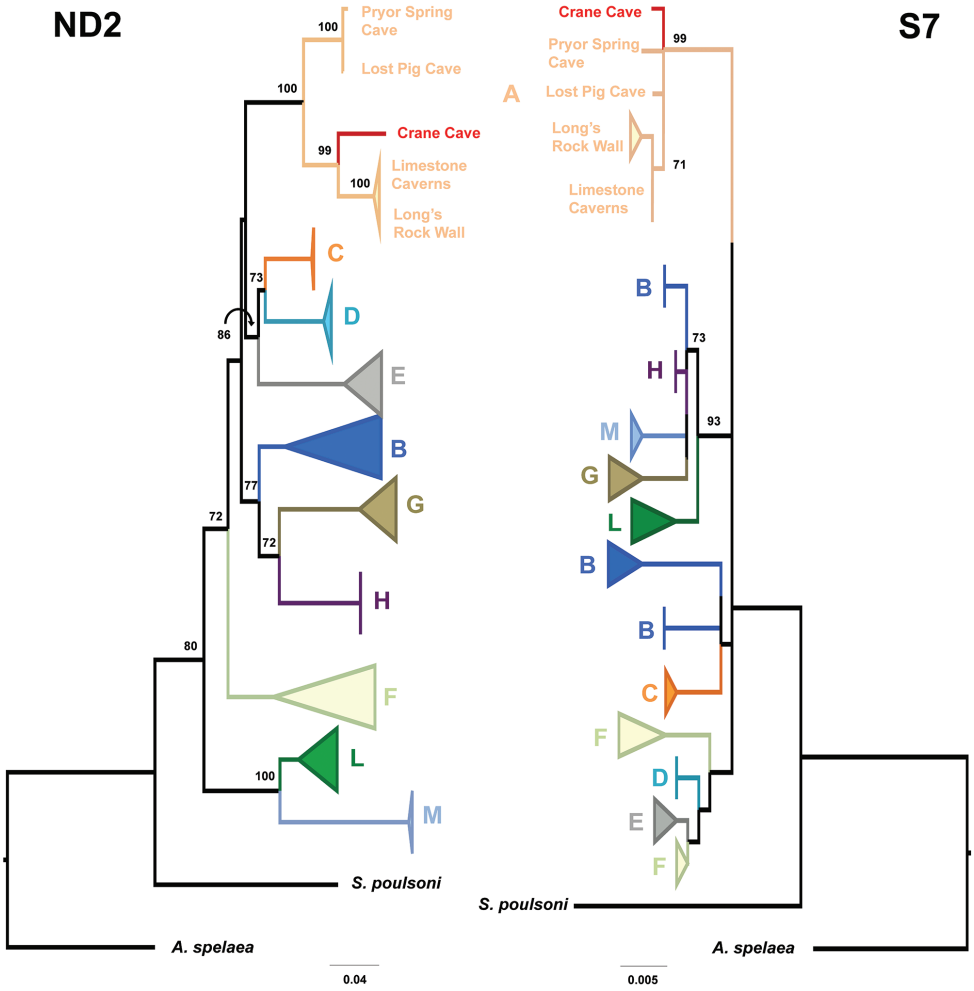

Figure 2. Maximum likelihood gene trees for mitochondrial ND2 (left) and nuclear S7 (right) loci. Colors correspond to genetic lineages for *Typhlichthys subterraenus* designated in Niemiller et al. (2012b). Bootstrap values are to the left (ND2) or right (S7) of the corresponding node with >0.70 support. Outgroup taxa include *Speoplatyrhinus poulsoni* and *Amblyopsis hoosieri*. Scale bar unit: expected substitutions per site.

Crane Cave (Fig. 3). The clade comprised of Crane Cave, Long's Rock Wall, and Limestone Caverns was sister to a population from Pryor Cave Spring (Tennessee Cave Survey no. TMN129) and Lost Pig Cave (TMN20) located in the Little Sequatchie River Valley and Sweetens Cove of southern Marion County, Tennessee, respectively. In contrast, the base of lineage A in the S7 phylogeny was a strongly supported polytomy that consisted of Crane Cave, Pryor Cave Spring, and a Long's Rock Wall + Limestone Caverns clade (Fig. 2). The ND2 and the S7 phylogenies both presented strong support for the monophyly of lineage A.

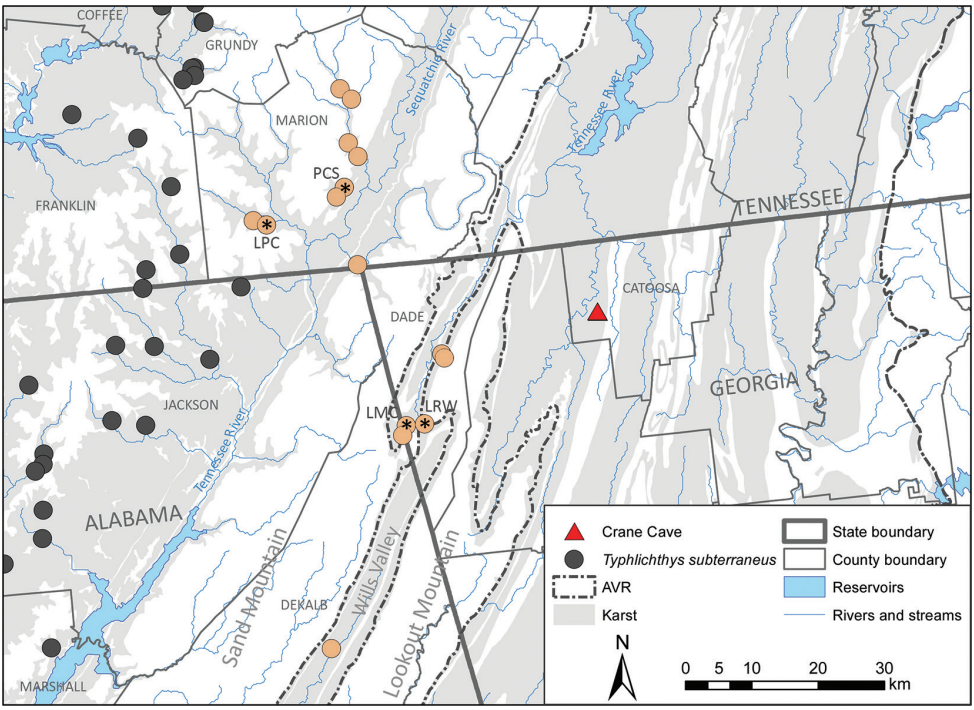

Figure 3. Distribution of *Typhlichthys subterraneus* (solid circles) in southeastern Tennessee, northeastern Alabama, and northwestern Georgia. The new record at Crane Cave is denoted with a red triangle and lineage A localities are highlighted in peach. Lineage A populations that have been genetically examined are marked with an asterisk and labeled as follows: LMC – Limestone Caverns, LPC – Lost Pig Cave, LRW – Long's Rock Wall, and PCS – Pryor Cave Spring. Counties with *Typhlichthys* records are labeled. Karst and cave-bearing strata are shaded gray based on the U.S. karst map (Weary and Doctor 2014). The border of the Appalachian Valley and Ridge (AVR) physiographic province is denoted by the dot and dashed line.

Discussion

The range of *Typhlichthys subterraneus s.l.* extends throughout the ILP of Kentucky, Tennessee, Alabama, and Georgia, which makes it one of the largest distributions of any cavefish in the world (Proudlove 2006, Niemiller and Poulson 2010). Because of the widespread distribution, even from distinct hydrological basins, several authors hypothesize that *T. subterraenus* represents a complex of morphologically cryptic, but genetically distinct, species (Swofford 1982; Barr and Holsinger 1985; Holsinger 2000; Niemiller and Fitzpatrick 2008; Niemiller and Poulson 2010). Niemiller et al. (2012b) identify at least ten cryptic lineages from a species delimitation analysis based on six loci and samples from 60 populations across the range. The most recent common ancestor of these lineages dates to the Late Pliocene to Early Pleistocene, about 2.8 million years ago (Mya) (95% confidence interval: 2.1–3.5 Mya; Niemiller et al. 2013a). Populations from Dade County, Georgia (Limestone Caverns and Long's Rock Wall), and at least two populations from the Little Sequatchie River Valley in Tennessee, form

a distinct genetic *Typhlichthys* lineage, referred to as lineage A. Populations that occur in Wills Valley in DeKalb County, Alabama, also are thought to belong to lineage A (Niemiller et al. 2013b), but have not been genetically examined to date. This lineage diverged from others in the ILP about 2.2 Mya (1.6–2.9 Mya based on 95% confidence intervals; Niemiller et al. 2012b, 2013a).

Analyses of the mitochondrial ND2 and the nuclear S7 loci from Crane Cave *T. subterraneus* strongly support affinity to lineage A (as defined by Niemiller et al. 2012b). However, the new Crane Cave record is ca. 24.2 km straight-line distance to the east from the next closest populations in Georgia and Alabama. Specifically, the *T. subterraneus* populations in Dade County are from caves formed in the Mississippian-age Bangor Limestone on the escarpments of Lookout Mountain and Fox Mountain, clearly within the Cumberland Plateau physiographic province. Despite the arbitrary boundary between the ILP and AVR, the distribution of lineage A now extends from the ILP into the Appalachians karst region because Crane Cave is well within the AVR and is from a hydrologically distinct watershed compared to the previously described *T. subterraneus* populations in the TAG region (Fig. 3).

There is the issue of whether the other *T. subterraneus* populations in lineage A, specifically those in Wills Valley formed in Cambrian-Ordovician Knox group dolomites in AVR-style structural valleys, are also considered AVR distributions or ILP distributions. The physiographic distinction of Wills Valley has been a matter of debate in the literature. Wills Valley is an anticlinal valley flanked by Sand Mountain to the west and Lookout Mountain to the east. Both ridges are considered parts of the Cumberland Plateau (Johnson 1930, Harkins et al. 1982, Raymond et al. 1988). As such, previous studies comparing subterranean biodiversity among karst regions have considered Wills Valley to be associated with the Cumberland Plateau and ILP karst region rather than the AVR within the Appalachians karst region (Peck 1989, 1995, Culver et al. 2003, Hobbs 2012). However, others have placed Wills Valley as part of the Ridge and Valley Level III ecoregion (Griffith et al. 2001) based on ecosystem similarity according to land use, geology, physiography, hydrology, climate, natural vegetation, and soils (Omernik 1987). Regardless, the distribution of *T. subterraneus* in Wills Valley caves and the evolution of the karst in the valley warrant further study. The transitional location of Wills Valley between the ILP and Appalachians karst region and its length (100+ km) may have been critical in the movement of *T. subterraneus* between the two larger karst regions.

Another important aspect of *T. subterraneus* in Wills Valley is that these populations are in the Coosa River watershed, which flows into the Alabama River and then Mobile Bay. Crane Cave occurs in the South Chickamauga Creek watershed, which flows into the Tennessee River. Moreover, all four documented populations in Dade County, Georgia, occur in the Lookout Creek watershed, and the caves in Marion County, Tennessee, are part of the Sequatchie River watershed. Both Lookout Creek and the Sequatchie River empty into the Tennessee River, which eventually flows into the Ohio River and then the Mississippi River. River drainages in the southern region of North America and the Appalachian Mountains became established at least by the Eocene, 55 Mya (Galloway et al. 2011, Hoagstrom et al. 2013). At this time, the ancestral Tennessee River and the Coosa River formed the Appalachian River that

flowed to Mobile Bay (Johnson 1905, Milici 1968). By the mid-Miocene through the Pliocene, uplift in the Southern Appalachians (Gallen et al. 2013) or of the Nashville Dome (Clark 1989), as well as potential regional base-level lowering, initiated downcutting by the ancestral Tennessee River through Walden Ridge and westward flow into the Sequatchie Valley, then around the Nashville Dome before being captured by the Ohio River (Milici 1968, Clark 1989, Self 2000). Some suggest that stream capture may have been facilitated by karst as "cavern capture" (*s.s.* Johnson 1905) in Walden Ridge and the Sequatchie Valley.

Today, the Tennessee and Coosa rivers are separated by a divide, whereby the southern part of Wills Valley flows to the Coosa River and the northern section flows to the Tennessee River. The genetic affiliation of the Crane Cave *T. subterraneus* population to lineage A (Niemiller et al. 2012b) suggests that this lineage has a shared evolutionary history, whereby a common ancestor must pre-date the emergence of the Tennessee-Coosa drainage divide and subsequent isolation of the Tennessee River from the Coosa River. The drainage divide likely formed in the late Pliocene based on evidence from changes in deltaic sedimentation (Galloway et al. 2011) and age dates from cave sediment records (Anthony and Granger 2007). This timeframe corresponds with the estimated divergence of lineage A from 2.9 to 1.6 Mya from other lineages in the ILP (Niemiller et al. 2012b, 2013a). Continued uplift and stream incision further isolated lineage A populations throughout the TAG region in the early Pleistocene, which resulted in genetically distinct populations in Crane Cave, Dade County, and Alabama/Tennessee.

In conclusion, although no additional cavefish populations have been discovered in the past several years (Freeman and Niemiller 2009), with the exception of Crane Cave, and despite several cave bioinventories and other studies in northwestern Georgia (Reeves et al. 2000, Buhlmann 2001, Miller and Niemiller 2008), the potential exists for additional *T. subterraneus* populations to be discovered. Caves to be targeted for exploration would be those within the South Chickamauga Creek watershed and formed in the Newala Limestone throughout Catoosa County, as well as extending southward into Walker County and northward into Hamilton County, Tennessee. Lastly, these *T. subterraneus* populations may provide insight into the geologic history of the Tennessee and Coosa rivers, as well as aid in the understanding of other endemic cave fauna in the TAG region. In particular, the boundary between the ILP and Appalachians karst regions (and Interior Plateau and AVR physiographic provinces) may not be as strong a barrier to dispersal for stygobiotic taxa as previously thought.

Acknowledgements

We particularly thank Mary and Ron Ziegler for allowing us to visit Crane Cave and for welcoming us on several return trips. Funding and support for this project was provided by the Cave Conservancy Foundation and the University of the South. We thank the Georgia Speleological Survey for providing data and a map of Crane Cave. This work was permitted by the Georgia Department of Natural Resources under scientific collection permit no. 8934 and approval by the University of the South IACUC committee (KSZ).

References

Barr TC, Holsinger JR (1985) Speciation in cave faunas. Annual Review of Ecology, Evolution, and Systematics 16: 313–337. doi: 10.1146/annurev.es.16.110185.001525

Buhlmann KA (2001) A biological inventory of eight caves in northwestern Georgia with conservation implications. Journal of Cave and Karst Studies 63: 91–98.

Christman MC, Culver DC (2001) The relationship between cave biodiversity and available habitat. Journal of Biogeography 28: 367–380. doi: 10.1046/j.1365-2699.2001.00549.x

Christman MC, Culver DC, Madden M, White D (2005) Patterns of endemism of the eastern North American cave fauna. Journal of Biogeography 32: 1441–1452. doi: 10.1111/j.1365-2699.2005.01263.x

Clark GM (1989) Central and Southern Appalachian water and wind gap origins: Review and new data. Geomorphology 2: 209–232. doi: 10.1016/0169-555X(89)90013-5

Cooper JE, Iles A (1971) The Southern Cavefish, *Typhlichthys subterraneus*, at the southeastern periphery of its range. National Speleological Society Bulletin 33: 45–49.

Cressler CW (1963) Geology and ground-water resources of Catoosa County, Georgia. Information Circular no. 28. The Geological Survey, Georgia State Division of Conservation, Atlanta, Georgia, 19 pp.

Cressler CW (1964) Geology and ground-water resources of Walker County, Georgia. Information Circular no. 29. The Geological Survey, Georgia State Division of Conservation, Atlanta, Georgia. 15 pp.

Croft MG (1964) Geology and ground-water resources of Dade County, Georgia. Information Circular no. 29. The Geological Survey, Georgia State Division of Conservation, Atlanta, Georgia, 17 pp.

Culver DC, Christman MC, Elliott WR, Hobbs HH III, Reddell JR (2003) The North American obligate cave fauna: regional patterns. Biodiversity and Conservation 12: 441–468. doi: 10.1023/A:1022425908017

Culver DC, Deharveng L, Bedos A, Lewis JJ, Madden M, Reddell JR, Sket B, Trontelj P, White D (2006) The midlatitude biodiversity ridge in terrestrial cave fauna. Ecography 29: 120–128. doi: 10.1111/j.2005.0906-7590.04435.x

Culver DC, Hobbs HH III, Mylroie JE (1999) Alabama: a subterranean biodiversity hotspot. Journal of the Alabama Academy of Science 70: 97–104.

Culver DC, Master LL, Christman MC, Hobbs HH III (2000) Obligate cave fauna of the 48 contiguous United States. Conservation Biology 14: 386–401. doi: 10.1046/j.1523-1739.2000.99026.x

Culver DC, Pipan T (2009) The biology of caves and other subterranean habitats. Oxford University Press, Oxford, 254 pp.

Eigenmann CH (1909) Cave Vertebrates of America. A Study in Degenerative Evolution. Carnegie Institution of Washington, Washington, 241 pp.

Freeman BJ, Niemiller ML (2009) Species profile for Southern Cavefish, *Typhlichthys subterraneus*. Georgia Rare Species Profiles. Georgia Department of Natural Resources, Wildlife Resources Division, Atlanta, Georgia.

Gallen SF, Wegmann KW, Bohnenstiehl DR (2013) Miocene rejuvenation of topographic relief in the southern Appalachians GSA Today 23: 4–10. doi: 10.1130/GSATG163A.1

Galloway WE, Whiteaker TL, Ganey-Curry P (2011) History of Cenozoic North American drainage basin evolution, sediment yield, and accumulation in the Gulf of Mexico basin. Geosphere 7: 938–973. doi: 10.1130/GES00647.1

Griffith GE, Omernik JM, Comstock JA, Lawrence S, Martin G, Goddard A, Hulcher, VJ, Foster T (2001) Ecoregions of Alabama and Georgia, (color poster with map, descriptive text, summary tables, and photographs). U.S. Geological Survey, Reston, Virginia.

Harkins JR (1982) Hydrology of Area 24, Eastern Coal Province, Alabama and Georgia. US Geological Survey Open-File Report 81-1113, 81 pp.

Hatcher RD Jr, Bream BR, Merschat AJ (2007) Tectonic map of the southern and central Appalachians: A tale of three orogens and a complete Wilson cycle. GSA Memoirs 200: 595–632. doi: 10.1130/2007.1200(29)

Hatcher RD Jr (2010) The Appalachian orogen: A brief summary. GSA Memoirs 206: 1–19. doi: 10.1130/2010.1206(01)

Hoagstrom CW, Ung V, Taylor K (2013) Miocene rivers and taxon cycles clarify the comparative biogeography of North American highland fishes. Journal of Biogeography 41: 644–658. doi: 10.1111/jbi.12244

Hobbs HH III (2012) Diversity patterns in the United States. In: White WB, Culver DC (Eds) Encyclopedia of caves (2nd edn). Academic Press, Amsterdam, 251–264. doi: 10.1016/b978-0-12-383832-2.00033-5

Holsinger JR (2000) Ecological derivation, colonization, and speciation. In: Wilkens H, Culver DC, Humphreys WF (Eds) Ecosystems of the World (Vol 30), Subterranean ecosystems. Elsevier, Amsterdam, 399–415.

Johnson DW (1905) The Tertiary history of the Tennessee River. Journal of Geology 13: 194–231. doi: 10.1086/621220

Johnson WD (1930) Physical divisions of northern Alabama (Bulletin 38). Geological Survey of Alabama, Tuscaloosa, Alabama, 48 pp.

Milici R (1968) Mesozoic and Cenozoic physiographic development of the Lower Tennessee River: In terms of the dynamic equilibrium concept. Journal of Geology 76: 472–479. doi: 10.1086/627345

Miller BT, Niemiller ML (2008) Distribution and relative abundance of Tennessee Cave Salamanders (*Gyrinophilus palleucus* and *G. gulolineatus*) with an emphasis on Tennessee populations. Herpetological Conservation and Biology 3: 1–20.

Niemiller ML, Fitzpatrick BM (2008) Phylogenetics of the Southern Cavefish (*Typhlichthys subterraneus*): implications for conservation and management. Proceedings of the 18th National Cave and Karst Management Symposium, St. Louis, Missouri, 79–88.

Niemiller ML, Poulson TL (2010) Subterranean fishes of North America: Amblyopsidae. In: Trajano E, Bichuette ME, Kappor BG (Eds) The biology of subterranean fishes. Science Publishers, Enfield, 169–280. doi: 10.1201/EBK1578086702-c7

Niemiller ML, Zigler KS (2013) Patterns of cave biodiversity and endemism in the Appalachians and Interior Plateau of Tennessee USA. PLoS ONE 8: e64177. doi: 10.1371/journal.pone.0064177

Niemiller ML, Fitzpatrick BM, Miller BT (2008) Recent divergence with gene flow in Tennessee Cave Salamanders (Plethodontidae: *Gyrinophilus*) inferred from gene genealogies. Molecular Ecology 17: 2258–2275. doi: 10.1111/j.1365-294X.2008.03750.x

Niemiller ML, Miller BT, Fitzpatrick BM (2009) Systematics and evolutionary history of subterranean *Gyrinophilus* salamanders. Proceedings of the 15th International Congress of Speleology, Kerrville, Texas 15: 242–248.

Niemiller ML, Fenolio DB, Zigler KS (2012a) The obligate cave fauna of Georgia. Bulletin of the Georgia Speleological Survey 2012: 6–12.

Niemiller ML, Near TJ, Fitzpatrick BM (2012b) Delimiting species using multilocus data: diagnosing cryptic diversity in the Southern Cavefish *Typhlichthys subterraneus* (Teleostei: Amblyopsidae). Evolution 66: 846–866. doi: 10.1111/j.1558-5646.2011.01480.x

Niemiller ML, Fitzpatrick BM, Shah P, Schmitz L, Near TJ (2013a) Evidence for repeated loss of selective constraint in rhodopsin of amblyopsid cavefishes (Teleostei: Amblyopsidae). Evolution 67: 732–748. doi: 10.1111/j.1558-5646.2012.01822.x

Niemiller ML, Graening GO, Fenolio DB, Godwin JC, Cooley JR, Pearson WR, Near TJ, Fitzpatrick BM (2013b) Doomed before they are described? The need for conservation assessments of cryptic species complexes using an amblyopsid cavefish (Amblyopsidae: *Typhlichthys*) as a case study. Biodiversity and Conservation 22: 1799–1820. doi: 10.1007/s10531-013-0514-4

Niemiller ML, Zigler KS, Fenolio DB (2013c) Cave life of TAG: a guide to commonly encountered species in Tennessee, Alabama, and Georgia. Biology Section of the National Speleological Society, Huntsville, Alabama, 45 pp.

Omernik JM (1987) Ecoregions of the conterminous United States. Annals of the Association of American Geographers 77: 118–125. doi: 10.1111/j.1467-8306.1987.tb00149.x

Peck SB (1989) The cave fauna of Alabama: part I. The terrestrial invertebrates (excluding insects). National Speleological Society Bulletin 51: 11–33.

Peck SB (1995) The cave fauna of Alabama: part II – The insects. National Speleological Society Bulletin 57: 1–19.

Poulson TL (1963) Cave adaptation in amblyopsid fishes. American Midland Naturalist 70: 257–290. doi: 10.2307/2423056

Proudlove GS (2006) Subterranean fishes of the world. International Society for Subterranean Biology, Moulis, 300 pp.

Raymond DE, Osborne WE, Copeland CW, Neathery TL (1988) Alabama stratigraphy. Circular 140. Geological Survey of Alabama, Stratigraphy and Paleontology Division, Tuscaloosa, 97 pp.

Reeves WK, Jensen JB, Ozier JC (2000) New faunal and fungal records from caves in Georgia, USA. Journal of Cave and Karst Studies 62: 169–179.

Self RP (2000) The pre-Pliocene course of the lower Tennessee River as deduced from river terrace gravels in southwest Tennessee. Southeastern Geology 39: 61–70.

Silvestro D, Michalak I (2012) raxmlGUI: a graphical front-end for RAxML. Organisms Diversity & Evolution 12: 335–337. doi: 10.1007/s13127-011-0056-0

Swofford DL (1982) Genetic variability, population differentiation, and biochemical relationships in the family Amblyopsidae. Master's Thesis, Eastern Kentucky University, Richmond, Kentucky.

Weary DJ, Doctor DH (2014) Karst in the United States: a digital map compilation and database. U.S. Geological Survey Open-File Report 2014-1156, 23 pp.

Subterranean Biology 20: 51–76 (2016)
doi: 10.3897/subtbiol.20.10389
http://subtbiol.pensoft.net

RESEARCH ARTICLE

A peer-reviewed open-access journal
Subterranean Biology
Published by The International Society for Subterranean Biology

Two new species of *Nitocrella* (Crustacea, Copepoda, Harpacticoida) from groundwaters of northwestern Australia expand the geographic range of the genus in a global hotspot of subterranean biodiversity

Danny Tang[1,2], Stefan M. Eberhard[1]

1 *Subterranean Ecology Pty Ltd, 227 Coningham Road, Coningham, Tasmania 7054, Australia* **2** *Environmental Laboratory and Ocean Monitoring Division, Orange County Sanitation District, 10844 Ellis Avenue, Fountain Valley, California 92708-7018, U.S.A.*

Corresponding author: *Stefan M. Eberhard* (stefan@subterraneanecology.com.au)

Academic editor: *O. Moldovan* | Received 2 September 2016 | Accepted 22 October 2016 | Published 10 November 2016

http://zoobank.org/CA9DCB75-8A1C-48BC-9AD5-B06403DAEFDD

Citation: Tang D, Eberhard SM (2016) Two new species of *Nitocrella* (Crustacea, Copepoda, Harpacticoida) from groundwaters of northwestern Australia expand the geographic range of the genus in a global hotspot of subterranean biodiversity. Subterranean Biology 20: 51–76. doi: 10.3897/subtbiol.20.10389

Abstract

In Australia, the Ameiridae is the most diverse harpacticoid family in groundwater, with 35 species hitherto reported. In this study, we describe two new species belonging to the "*vasconica*"-group of the ameirid genus *Nitocrella* based on specimens collected from groundwaters near mine sites in the Pilbara and Great Sandy Desert regions of northwestern Australia. *Nitocrella knotti* **sp. n.** can be distinguished from related taxa by having two setae on the antennal exopod, four armature elements on the distal endopodal segment of leg 2, four armature elements on the distal endopodal segment of leg 3, three armature elements on the distal endopodal segment of leg 4, and three setae on the basoendopodal lobe of leg 5. *Nitocrella karanovici* **sp. n.** differs from its congeners by having a short outer spine and long inner seta on the distal endopodal segment of leg 2, three armature elements on the distal endopodal segment of leg 3, and four setae on the basoendopodal lobe of leg 5 in the female. This study is of biogeographic interest in providing the first documentation of the genus *Nitocrella* from the Pilbara and Great Sandy Desert regions. Both new species of *Nitocrella* are recorded from restricted localities and appear to be short-range endemics, thus making them potentially vulnerable to environmental changes and threatening processes such as mining. The distribution range of *N. karanovici* **sp. n.** coincides with the centre of diversity of the Ethel Gorge aquifer stygobiont community, a globally significant hotspot which is listed as endangered.

Keywords
Short-range endemic, stygofauna, compliance monitoring, mining

Introduction

The family Ameiridae (Copepoda) has successfully colonized and radiated in continental surface water and groundwater, with over 150 species reported from Australia, Asia, Europe, and North America (Boxshall and Halsey 2004, Boxshall and Defaye 2008). In Australia, the Ameiridae is the most diverse harpacticoid family in groundwater (Karanovic 2006, Karanovic and Hancock 2009). Presently 35 ameirid species have been reported from groundwater, mostly in Western Australia and a few in Queensland and South Australia (Table 1).

Groundwaters of (semi-) arid Western Australia are a globally significant hotspot for subterranean biodiversity (Humphreys 2008, Eberhard et al. 2009, Guzik et al. 2011). Most of this rich diversity occurs in two adjacent geographic regions, the Pilbara and the northern Yilgarn, both of which form parts of the Western Shield, a single emergent land mass since the Proterozoic (Fig. 1A). In both regions, progressive Quaternary climatic aridity is considered the major driver for groundwater colonization; however, there are some remarkable differences between the copepod and other stygofauna taxa of the Pilbara and northern Yilgarn, which have almost no genera in common and exhibit major differences in higher taxa (Karanovic 2006, Humphreys 2008, 2012). An explanation for this great biogeographic disjunction remains elusive.

The majority of ameirid species known from groundwater in Australia were collected at, or adjacent to, proposed mine sites which have been surveyed for stygofauna as part of the mine project environmental impact assessment, or, as part of the ongoing environmental compliance monitoring at established mine sites. Other ameirids were collected from pastoral wells and groundwater boreholes during the course of surveys by government departments, museums, and universities. Most of the described ameirids are recorded from single localities and appear to be short-range endemic species (sensu Harvey 2002, Harvey et al. 2011), and this makes them potentially vulnerable to environmental changes and threatening processes such as mining and groundwater abstraction. In some cases this has led to conflict between the competing interests of biodiversity conservation and resources development (see Karanovic et al. 2013). In this study, we describe two new ameirid species belonging to the genus *Nitocrella* Chappuis, 1923 based on specimens collected from groundwaters near two mine sites in the Pilbara and Great Sandy Desert regions of northwestern Western Australia.

Methods

The net-haul method (see Eberhard et al. 2005, 2009) was used to collect samples of stygofauna in July 2008 from one borehole located 48 km from the Telfer Mine in

Table 1. Harpacticoid copepods of the family Ameiridae reported from subterranean waters of Australia (in alphabetical order).

Species	State*	Reference
Abnitocrella eberhardi Karanovic, 2006	WA	Karanovic (2006)
Abnitocrella halsei Karanovic, 2006	WA	Karanovic (2006)
Antistygonitocrella pardalotos Karanovic, Eberhard, Perina & Callan, 2013	WA	Karanovic et al. (2013)
Archinitocrella newmanensis Karanovic, 2006	WA	Karanovic (2006)
Biameiropsis barrowensis Karanovic, 2006	WA	Karanovic (2006)
Gordanitocrella trajani Karanovic & Hancock, 2009	WA	Karanovic and Hancock (2009)
Haifameira pori Karanovic, 2004	WA	Karanovic (2004)
Hirtaleptomesochra bispinosa Karanovic, 2004	WA	Karanovic (2004)
Inermipes humphreysi Lee & Huys, 2002	WA	Lee and Huys (2002)
Kimberleynitocrella billhumphreysi Karanovic & Hancock, 2009	WA	Karanovic and Hancock (2009)
Lucionitocrella yalleenensis Karanovic & Hancock, 2009	WA	Karanovic and Hancock (2009)
Megastygonitocrella bispinosa (Karanovic, 2006)	WA	Karanovic (2006), Karanovic and Hancock (2009)
Megastygonitocrella dec Karanovic & Hancock, 2009	WA	Karanovic and Hancock (2009)
Megastygonitocrella ecowisei Karanovic & Hancock, 2009	WA	Karanovic and Hancock (2009)
Megastygonitocrella embe Karanovic, Eberhard, Perina & Callan, 2013	WA	Karanovic et al. (2013)
Megastygonitocrella kryptos Karanovic & Hancock, 2009	QLD	Karanovic and Hancock (2009)
Megastygonitocrella pagusregalis Karanovic & Hancock, 2009	QLD	Karanovic and Hancock (2009)
Megastygonitocrella trispinosa (Karanovic, 2006)	WA	Karanovic (2006), Karanovic and Hancock (2009)
Megastygonitocrella unispinosa (Karanovic, 2006)	WA	Karanovic (2006), Karanovic and Hancock (2009)
Nitocrella absentia Karanovic, 2004	WA	Karanovic (2004)
Nitocrella obesa Karanovic, 2004	WA	Karanovic (2004)
Nitocrella trajani Karanovic, 2004	WA	Karanovic (2004)
Nitocrellopsis halsei Karanovic, 2010	WA	Karanovic (2010)
Nitocrellopsis operculata Karanovic, 2010	WA	Karanovic (2010)
Nitocrellopsis pinderi Karanovic, 2010	WA	Karanovic (2010)
Nitokra esbe Karanovic, Eberhard, Cooper & Guzik, 2014	WA	Karanovic et al. (2014)
Nitokra humphreysi Karanovic & Pesce, 2002	WA	Karanovic and Pesce (2002)
Nitokra lacustris (Schmankevitsch, 1895)	SA	Zeidler (1989)
Nitokra lacustris pacifica Yeatman, 1983	WA	Karanovic (2004), Tang and Knott (2009)
Nitokra yeelirrie Karanovic, Eberhard, Cooper & Guzik, 2014	WA	Karanovic et al. (2014)
Novanitocrella aboriginesi Karanovic, 2004	WA	Karanovic (2004)
Paranitocrella bastiani Tang & Knott, 2009	WA	Tang and Knott (2009)
Parapseudoleptomesochra karamani Karanovic, 2004	WA	Karanovic (2004)
Parapseudoleptomesochra rouchi Karanovic, 2004	WA	Karanovic (2004)
Parapseudoleptomesochra tureei Karanovic, 2006	WA	Karanovic (2006)

*WA = Western Australia; QLD = Queensland; SA = South Australia

the Great Sandy Desert region of Western Australia, and annually, from 2009–2012, from a total of eight boreholes in the Ethel Gorge aquifer adjacent to two mine pits, namely Orebody 23 and Orebody 25, 15 km NE of Newman in the Pilbara region of Western Australia (Fig. 1). Each net-haul sample was transferred to a labelled 33 ml

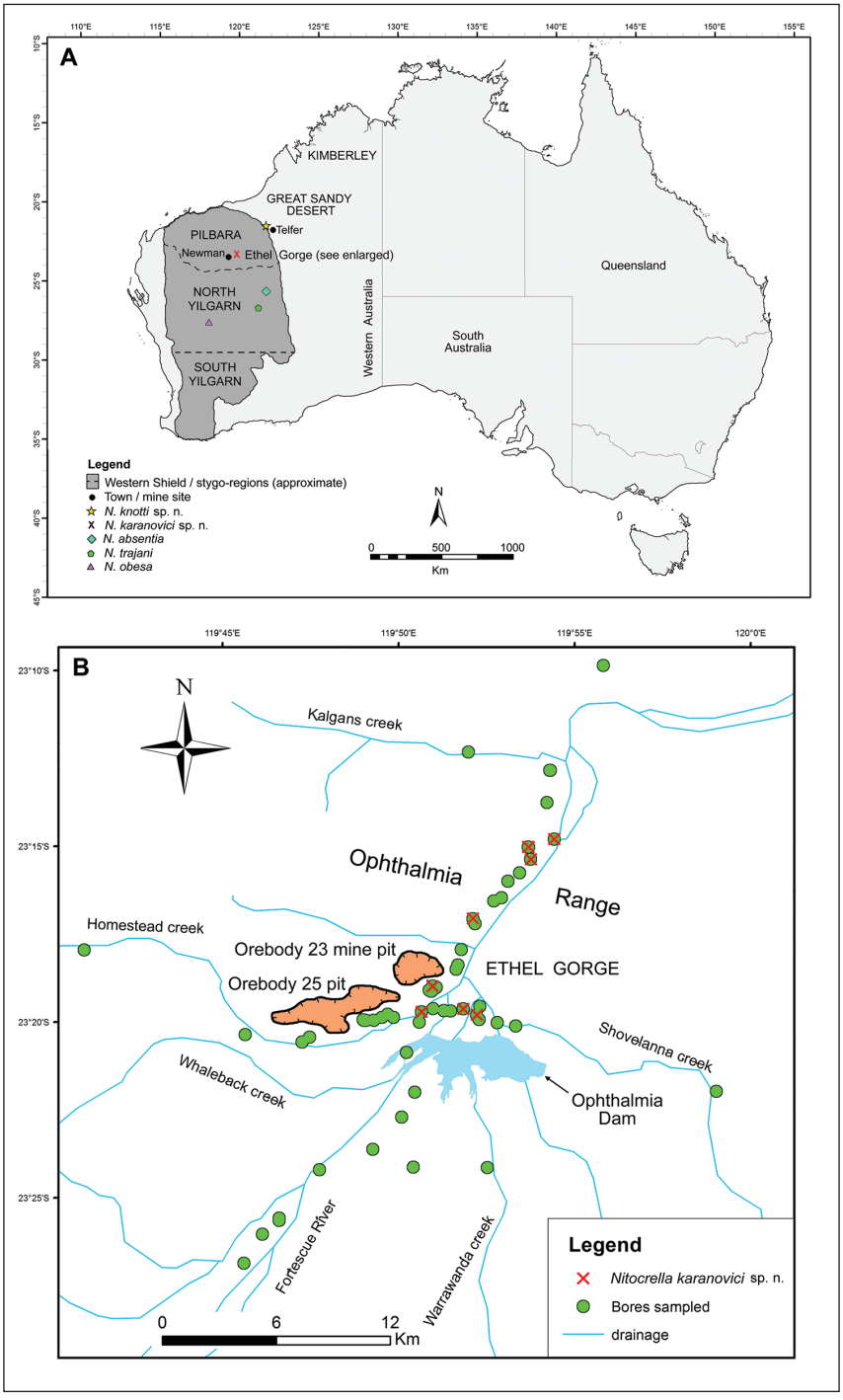

Figure 1. A Map showing the species of *Nitocrella* reported from Western Australia **B** Enlarged map of the Ethel Gorge area showing sampled boreholes and collection sites for *Nitocrella karanovici* sp. n. in relation to surface drainage and mine pits.

vial and preserved in absolute alcohol for possible future molecular studies (although molecular studies were not required for this taxonomic description of *Nitocrella* species which were well defined on the basis of morphology). Samples were transported to the laboratory where copepods were sorted from debris and other stygofauna using a dissecting microscope.

Copepod specimens selected for taxonomic study were soaked in lactophenol prior to examination using a Leica M205C dissecting microscope and Leica MD2500 compound microscope equipped with differential interference contrast. Selected specimens were measured using an ocular micrometer, dissected, and examined using the wooden slide procedure of Humes and Gooding (1964). Selected whole specimens and dissected appendages were also drawn with the aid of a drawing tube. Morphological terminology follows Huys and Boxshall (1991). Type material was deposited in the Western Australian Museum (WAM), Kewdale, Australia.

Results

Order Harpacticoida Sars, 1903
Family Ameiridae Boeck, 1865
Genus *Nitocrella* Chappuis, 1923

***Nitocrella knotti* sp. n.**
http://zoobank.org/A2B3DDF8-47A5-4435-B405-5AF902BF18BA
Figs 2–4

Type locality. Borehole HB54/4.1 (= Borehole HB405) (21°28'45"S; 121°46'45"E) on Telfer Road, 48 km NW of Telfer mine site and 350 km SE of Port Hedland, Western Australia, July 2008, J. Mifsud leg.

Type material. Holotype female (WAM C51830) in absolute alcohol, 1 female paratype (WAM C51831) dissected and mounted on a slide, and 4 female paratypes (WAM C51832) in absolute alcohol.

Description. *Female*. Body (Fig. 2A) subcylindrical, 610–635 μm (mean 620 μm; n = 3) long (measured from tip of rostrum to posterior margin of caudal rami) and 148–155 μm (mean 153 μm; n = 3) wide (at posterolateral margin of cephalothorax). Prosome composed of cephalothorax and 3 free pedigerous somites; tergite of first two pedigerous somites each with elliptical integumental window. Urosome comprised of fifth pedigerous somite, genital double-somite, and 3 free abdominal somites. Fifth pedigerous somite with short, dorsolateral row of spinules and numerous short rows of minute denticles (not drawn) and minute surface pits (not drawn) on dorsal and ventral surfaces. Components of genital double-somite (Fig. 2A, B) partially fused dorsally but completely fused ventrally, ornamented with short, anterolateral row of spinules on ventral surface, minute surface pits and numerous short rows of minute denticles on dorsal and ventral surfaces (only minute denticles on ventral surface are

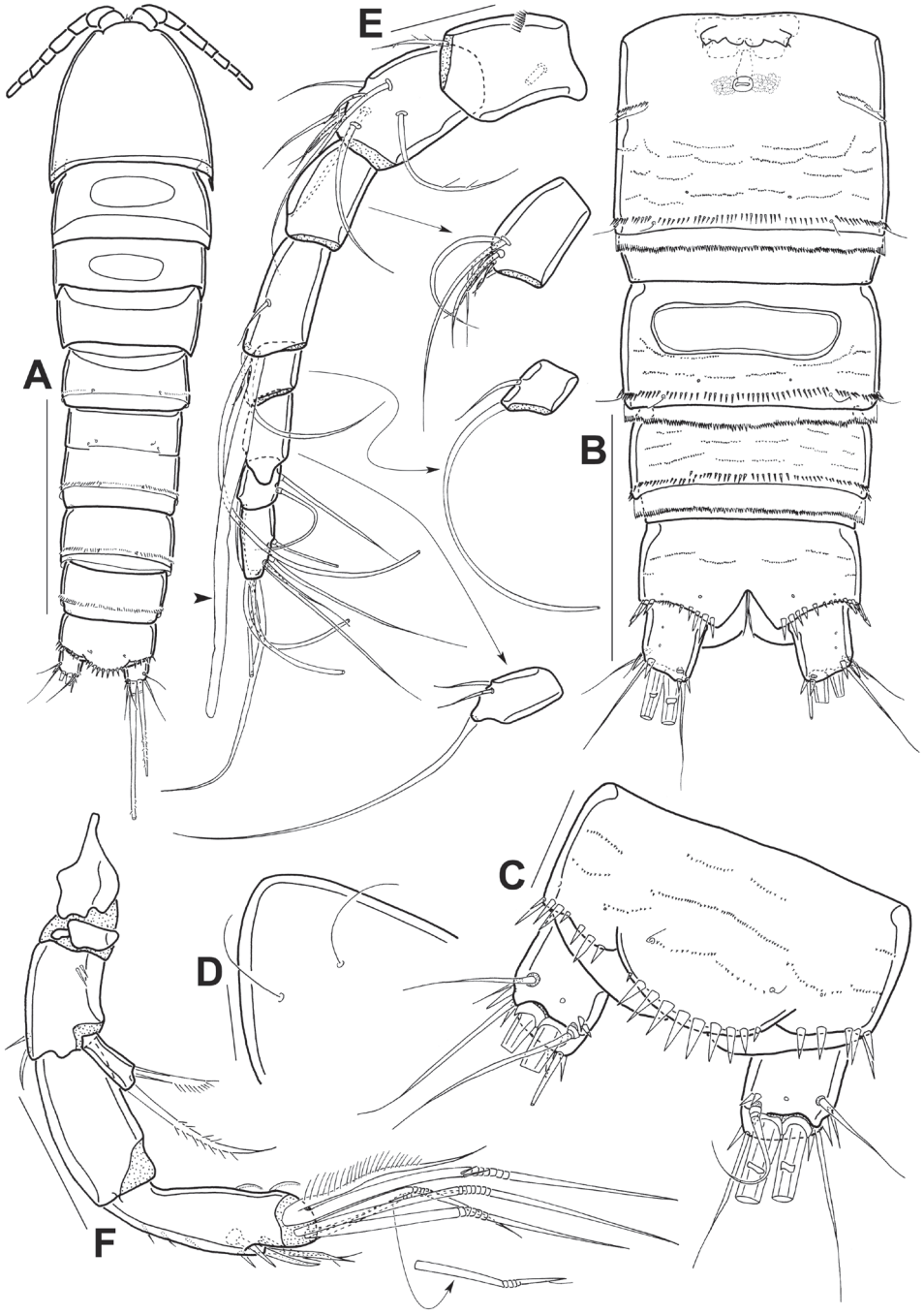

Figure 2. *Nitocrella knotti* sp. n., adult female: **A** habitus, dorsal **B** urosomites 2–5 and caudal rami, ventral **C** anal somite and caudal rami, dorsal **D** rostrum, dorsal **E** right antennule with segments 3, 5 and 6 shown separately and aesthetasc indicated by arrowhead, ventral **F** left antenna with one apical element shown separately, anterior. Scale bars: **A** 200 μm; **B** 100 μm; **C, E, F** 25 μm; **D** 2 μm.

shown), and row of large spinules and serrated hyaline frill encircling posterior margin; genital field with large median copulatory pore, chitinized copulatory duct leading anteriorly to pair of bilobate seminal receptacles, and median genital pore covered by operculiform leg 6. First free abdominal somite with large integumental window on ventral surface, minute surface pits and numerous short rows of minute denticles on dorsal and ventral surfaces (only minute denticles on ventral surface are shown), and row of large spinules and serrated hyaline frill ringing posterior margin. Second free abdominal somite similar to preceding somite, but without integumental window. Anal somite (Fig. 2B, C) with minute surface pits (not drawn) and numerous short rows of minute denticles on dorsal and ventral surfaces and row of large spinules along posterior border and along posterior margin of anal operculum.

Caudal ramus (Fig. 2B, C) short, about 1.25 times longer than wide, bears minute surface pits (not drawn) and 7 setae. Insertion point of setae III, VI and VII flanked by spinules. Setae IV and V spinulate, with proximal breaking planes; other setae naked. Seta VII basally tri-articulate.

Rostrum (Fig. 2D) subtrianglar, not demarcated at base, with 2 dorsal sensilla.

Antennule (Fig. 2E) 8-segmented, with armature as follows: 1, 9, 8, 4 + ae, 2, 3, 4, and 8. Segment 1 proximally with additional spinular row and large tubular pore. Two (of 4) and 4 (of 8) setae basally biarticulate on segments 7 and 8, respectively. Two (of 4) anterodistal setae on segment 8 fused at base.

Antenna (Fig. 2F), comprising coxa, basis, exopod, and 2-segmented endopod. Coxa naked and unarmed. Basis with 2 small proximal spinules and 2 large inner distal spinules. Exopod 1-segmented, cylindrical, and armed with 2 pinnate setae. Proximal endopodal segment naked and unarmed. Distal endopodal segment as long as basis and proximal endopodal segment combined; ornamented with 2 distolateral hyaline frills, 3 proximomedial spinules, and 2 distomedial spinules; armed with 2 spines (1 spine with minute spinules along inner margin; other with subapical flagellum) plus 2 naked setae along inner subdistal margin and 1 pilose and 5 geniculate setae along apical margin (lateralmost geniculate seta with 2 spinules at mid-point and fused basally with pilose seta; shortest geniculate seta with subapical flagellum).

Labrum (Fig. 3A) subtriangular, with denticles along apical margin and large distolateral denticles plus 2 patches of minute denticles on posterior face.

Mandible (Fig. 3B) composed of coxa and 2-segmented palp. Coxa with inner subapical process, numerous unicuspidate teeth along distal margin, and unilaterally denticulate seta on inner distal angle. Proximal segment of palp unarmed, but furnished with 2 proximomedial spinules, 1 medial process, and row of apical spinules; distal segment armed with 5 apical naked setae.

Maxillule (Fig. 3C) composed of praecoxa and 3-segmented palp. Praecoxal arthrite bears proximal crescentic row of spinules, 2 chitinized naked setae on inner subapical margin, 2 long naked setae on anterior surface, and 8 apical elements (3 highly chitinized, of which 2 each furnished with minute apical teeth; 1 unipinnate; 1 with bristled tip; 3 naked). Coxal endite elongated, with subapical row of spinules and 1 geniculate and 2 naked setae at distal end. Basis as long as coxa, bears 5 apical

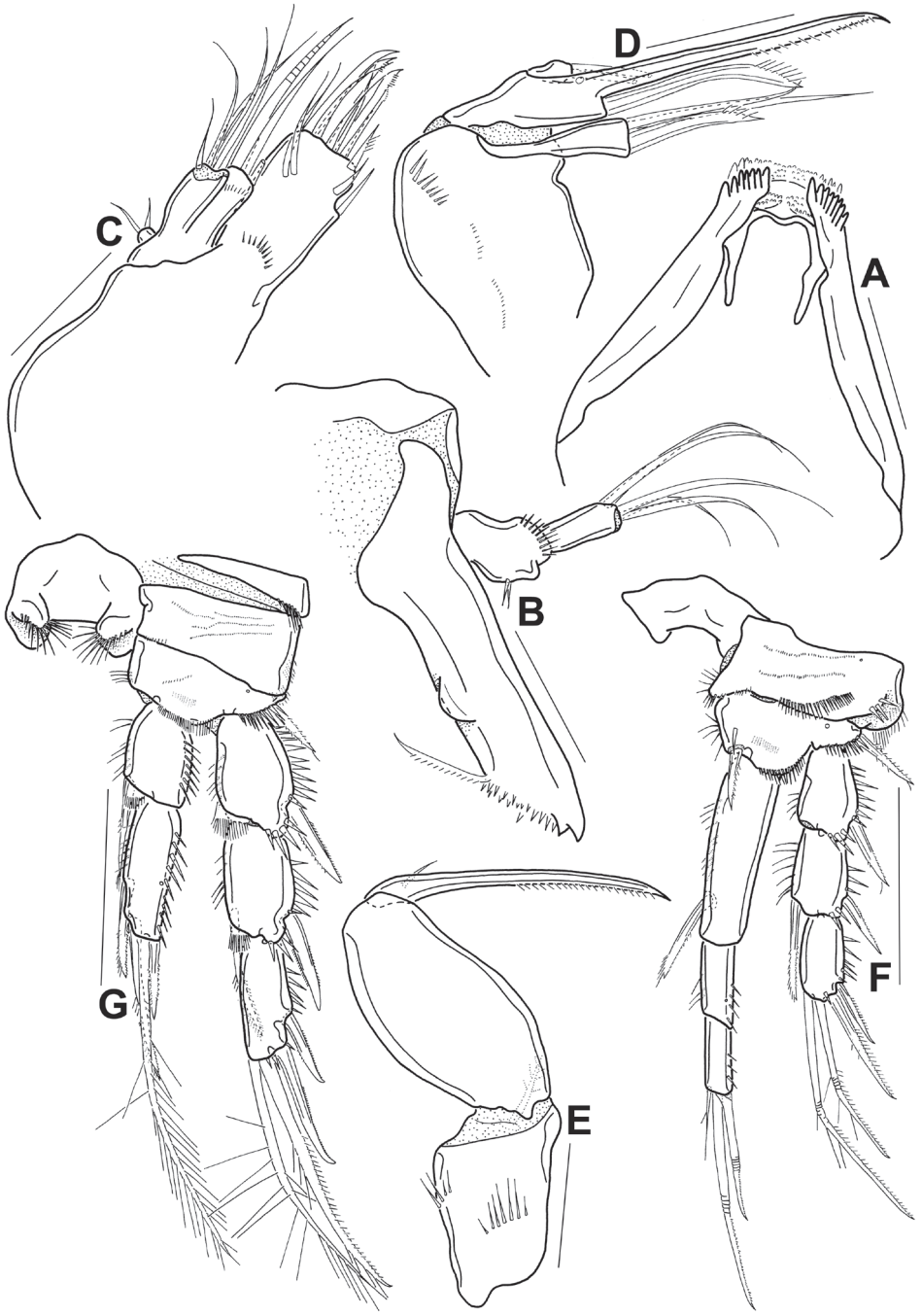

Figure 3. *Nitocrella knotti* sp. n., adult female: **A** labrum, posterior **B** left mandible, anterior **C** left maxillule, anterior **D** left maxilla, anterior **E** right maxilliped, posterior **F** left leg 1, anterior **G** left leg 2, anterior. Scale bars: **A, B, C, D, E** 20 μm; **F, G** 50 μm.

naked setae. Endopod 1-segmented, vestigial, armed with 2 short naked setae. Exopod absent.

Maxilla (Fig. 3D) 3-segmented, composed of syncoxa, allobasis, and 1-segmented endopod. Syncoxa large, with 3 longitudinal rows of spinules on anterior surface and 1 pectinate and 2 naked apical setae on distal endite. Allobasis drawn out into long claw furnished with spinules along distal half of inner margin and bears 1 pectinate seta. Endopod 1-segmented, armed with 2 long apical setae.

Maxilliped (Fig. 3E) 3-segmented, comprising syncoxa, basis, and 1-segmented endopod. Syncoxa stout, with 2 rows of long spinules and 1 distal pinnate seta. Basis naked and unarmed, about 1.5 times as long as syncoxa. Endopod drawn out into long claw, with 1 proximal naked seta and denticles along distal half of inner margin.

Legs 1–4 biramous (Figs 3F, G, 4A, B); leg 1 with trimerous rami; legs 2–4 with trimerous exopod and bimerous endopod. Armature on rami of legs 1 to 4 as follows (Roman numerals = spines; Arabic numerals = setae):

	Coxa	**Basis**	**Exopod**	**Endopod**
Leg 1	0-0	I-I	I-0; I-I; II,2,0	0-I; 0-0; 0,3,0
Leg 2	0-0	I-0	I-0; I-I; II,2,0	0-I; 0,I+1,II
Leg 3	0-0	1-0	I-0; I-I; II,2,0	0-I; 0,I+1,1+I
Leg 4	0-0	1-0	I-0; I-I; II,2,2	0-I; 0,I+1,I

Leg 1 (Fig. 3F) intercoxal sclerite naked and about twice as wide as long. Coxa with 2 rows of minute spinules and 1 row of large spinules on anterior surface; outer margin with 1 row of large spinules and 2 rows of fine spinules; inner distal corner with row of fine spinules. Basis with row of large spinules at insertion of each ramus and row of fine spinules along inner margin and on posterior surface; 1 additional large spinule present near base of inner spine; both spines with subapical flagellum. Outer spine on proximal exopodal segment with subapical flagellum. First two exopodal segments with large spinules along outer margin and on outer distal corner, as well as fine spinules along inner margin; distal segment with large spinules along outer margin and 1 spinule on apical margin. Both setae on terminal exopodal segment geniculate. Proximal endopodal segment long, extending almost to mid-point of distal exopodal segment, with fine spinules along inner margin; middle segment with fine spinules along outer and inner margins; distal segment with fine spinules along outer margin. Two (of 3) setae on distal endopodal segment geniculate. Both rami with minute surface pits (not drawn).

Leg 2 (Fig. 3G) intercoxal sclerite posteriorly bilobate, with row of spinules on each lobe. Coxa with 4 rows of minute spinules on anterior surface; outer margin with 2 rows of fine spinules; inner distal corner with row of minute spinules. Basis ornamented as in leg 1, except lacks large spinule near inner margin and with additional row of minute spinules on anterior surface. Exopod ornamented as in leg 1, except with additional spinulated frill on inner distal corner of proximal and middle segments. First two exopodal segments protruded on outer distal corner. Proximal endopodal segment with spinules along outer and inner margins and short spinulated frill on

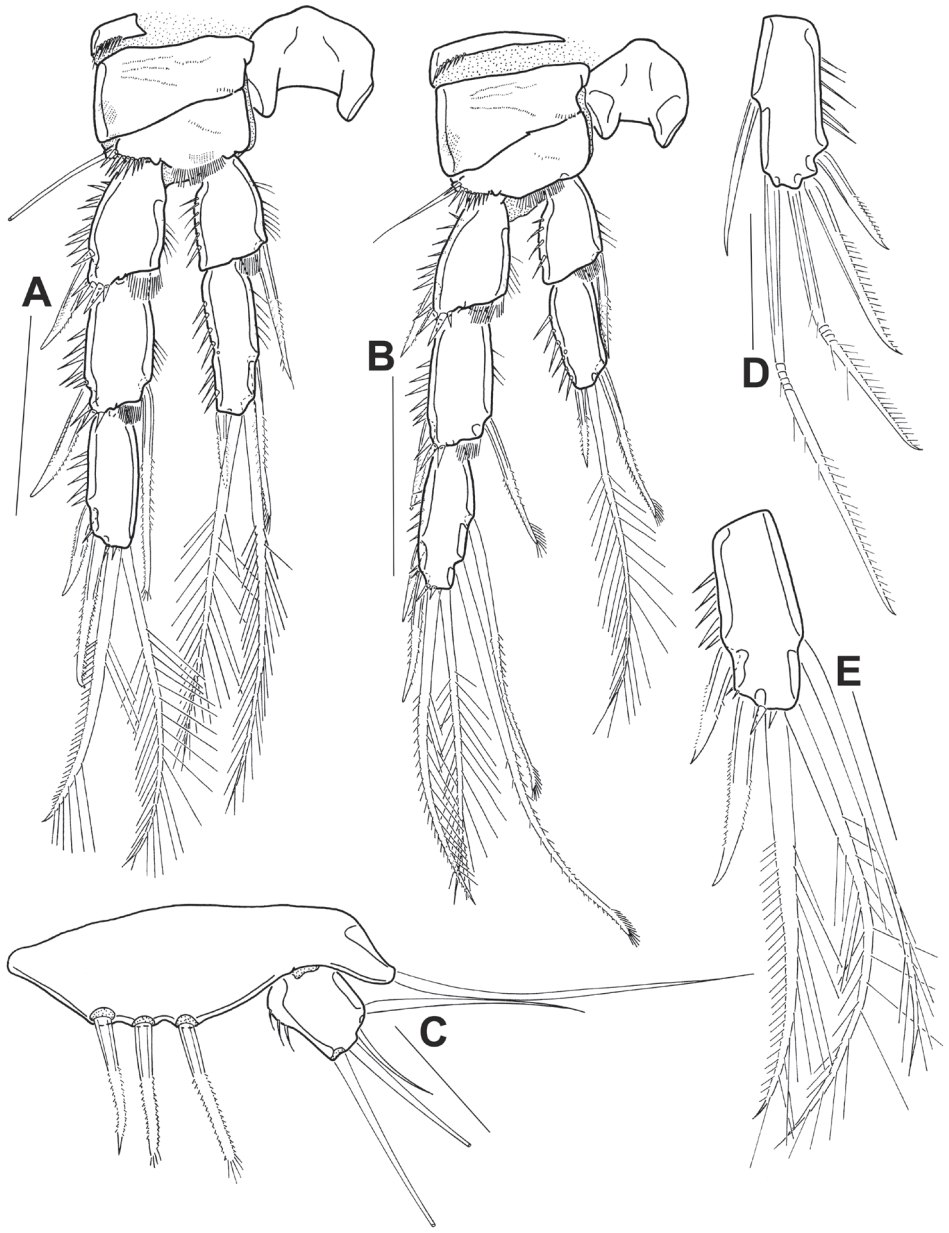

Figure 4. *Nitocrella knotti* sp. n., adult female: **A** right leg 3, anterior **B** right leg 4, anterior **C** left leg 5, ventral **D** terminal exopodal segment of left leg 1, anterior **E** terminal exopodal segment of right leg 2, anterior. Scale bars: **A, B** 50 μm; **C, D** 20 μm; **E** 25 μm.

inner distal corner. Distal endopodal segment about 1.5 times longer than proximal endopodal segment and furnished with spinules along outer and inner margins. Both rami with minute surface pits (not drawn) as in leg 1.

Leg 3 (Fig. 4A) similar to leg 2, except with naked intercoxal sclerite, outer seta (instead of spine) on basis, longer inner spine on middle exopodal segment, and inner distal seta (instead of spine) and longer inner proximal spine on distal endopodal segment.

Leg 4 (Fig. 4B) similar to leg 3, except with smaller intercoxal sclerite, row of spinules absent on posterior surface of basis, less protruded outer distal corner on first two exopodal segments, shorter inner spine on middle exopodal segment, 2 more elements on distal exopodal segment, shorter distal endopodal segment, and only 3 elements on distal endopodal segment.

Leg 5 (Fig. 4C) biramous. Basoendopod with long outer basal seta and 3 spinulated setae on endopodal lobe. Exopod 1-segmented, slightly longer than wide, with few spinules along inner margin and 4 unequal naked setae.

Leg 6 (Fig. 2B) represented by simple operculum covering genital pore, armed with 1 minute naked seta on outer distal corners.

Male. Unknown.

Variability. One paratype with 5 elements on terminal exopodal segment of left leg 1 (Fig. 4D). Another paratype with 5 elements on terminal exopodal segment of right leg 2 (Fig. 4E).

Etymology. This species is named in honour of the late Professor Brenton Knott (The University of Western Australia) who made significant contributions to research on groundwater fauna in Western Australia.

Differential diagnosis. Among the three groups of *Nitocrella* proposed by Petkovski (1976), i.e. "*chappuisi*", "*hirta*", and "*vasconica*", *Nitocrella knotti* sp. n. belongs to the "*vasconica*"-group as it also possesses the characteristic six armature elements on the distal exopodal segment of leg 4. With the addition of *N. knotti* sp. n. (including the second new species described below), this group currently contains 21 species reported from Eurasia, the Caribbean, and Australia (Table 2). *Nitocrella knotti* sp. n. shares with *N. afghanica* Štěrba, 1973, *N. jankowskajae* Borutzky, 1972, *N. kirgizica* Borutzky, 1972, *N. monchenkoi* Borutzky, 1972, *N. obesa* Karanovic, 2004, and *N. trajani* Karanovic, 2004 an armature formula of I-0; I-I; II,2,0 on the exopod and 0-I; 0-0; 0,3,0 on the endopod of leg 1, II,2,0 on the distal exopodal segment of legs 2 and 3, and 0-I on the proximal endopodal segment of legs 2–4. However, *N. knotti* sp. n. can be easily distinguished from those taxa by having four armature elements (instead of two for *N. jankowskaja*, or three for *N. afghanica*, *N. kirgizica*, *N. monchenkoi*, *N. obesa*, and *N. trajani*) on the distal endopodal segment of leg 2. *Nitocrella knotti* sp. n. differs further from the Australian *N. obesa* and *N. trajani* by having the genital and first abdominal somites fused ventrally (rather than completely separate), two setae (rather than three) on the antennal exopod, and three setae (rather than four) on the basoendopodal lobe of leg 5, among others; and from the Central Asian *N. afghanica*, *N. jankowskajae*, *N. kirgizica*, and *N. monchenkoi* by having four armature elements (instead of two) on the distal endopodal segment of leg 3 and three armature elements (instead of one for *N. afghanica*, or two for *N. jankowskaja*, *N. kirgizica*, and *N. monchenkoi*) on the distal endopodal segment of leg 4.

Table 2. Species of *Nitocrella* belonging to the "*vasconica*"-group (in alphabetical order).

Species	Locality	Reference
N. absentia Karanovic, 2004	Australia	Karanovic (2004)
N. afghanica Štěrba, 1973	Afghanistan	Štěrba (1973)
N. beatricis Cottarelli & Bruno, 1993	Sardinia (Italy); Corsica (France)	Cottarelli and Bruno (1993)
N. caraioni Petkovski, 1976	Cuba	Petkovski (1976)
N. dussarti Chappuis & Rouch, 1959	Pyrenees (France)	Chappuis and Rouch (1959)
N. gracilis Chappuis, 1955	Pyrenees (France)	Chappuis (1955), Rouch (1964)
N. knotti sp. n.	Australia	Present study
N. jankowskajae Borutzky, 1972	Kirgiziya (= Kyrgyzstan)	Borutzky (1972)
N. karanovici sp. n.	Australia	Present study
N. kirgizica Borutzky, 1972	Kirgiziya (= Kyrgyzstan)	Borutzky (1972)
N. mara Löffler, 1959	Iran	Löffler (1959)
N. monchenkoi Borutzky, 1972	Uzbekistan	Borutzky (1972)
N. motasi Petkovski, 1976	Cuba	Petkovski (1976)
N. nana Štěrba, 1973	Afghanistan	Štěrba (1973)
N. obesa Karanovic, 2004	Australia	Karanovic (2004)
N. paceae Pesce, 1980	Iran	Pesce (1980)
N. stetinai Štěrba, 1973	Afghanistan	Štěrba (1973)
N. trajani Karanovic, 2004	Australia	Karanovic (2004)
N. unispinosa Shen & Tai, 1973	China	Shen and Tai (1973)
N. vasconica Chappuis, 1937	Spain	Chappuis (1937)
N. yokotai Miura, 1962	Japan	Miura (1962)

Nitocrella karanovici sp. n.

http://zoobank.org/BF208E60-B0C2-4AF1-9857-04716000206E
Figs 5–9

Type locality. Borehole UNK02 (23°15'00"S; 119°53'41"E), Ethel Gorge aquifer, approximately 15 km ENE of Newman (Fig. 1), Western Australia, 8 February 2011, P. Bell and S. Catomore leg.

Type material. Holotype female (WAM C51837) in absolute alcohol, allotype male (WAM C51838) in absolute alcohol, 4 paratype females and 2 paratype males (WAM C51839–C51844) dissected and mounted on one slide each, and 26 paratype females, 28 paratype males and 1 copepodid (WAM C51845) in absolute alcohol.

Other material examined. All material collected from boreholes in the Ethel Gorge aquifer, approximately 15 km ENE of Newman, Western Australia (Fig. 1). 3 females and 1 male (WAM C51833) in absolute alcohol, borehole EEX917 (23°19'47"S; 119°52'13"E), 8 February 2012, S. Catomore and N. Coen leg.; 7 females (WAM C51834) in absolute alcohol, borehole EEX917 (23°19'47"S; 119°52'13"E), 12 April 2012, P. Bell and S. Catomore leg.; 1 male (WAM C51835) in absolute alcohol, borehole P13S (23°18'56"S; 119°50'58"E), 21 November 2009, P. Bell and G. Perina leg.; 1 female (WAM C51836) in absolute alcohol, borehole T399 (23°17'03"S;

119°52'07"E), 5 November 2010, P. Bell and S. Catomore leg.; 13 females and 3 males (WAM C51846) in absolute alcohol, borehole UNK02 (23°15'00"S; 119°53'41"E), 9 February 2011, P. Bell and S. Catomore leg.; 2 females (WAM C51847) in absolute alcohol, borehole W105 (23°19'37"S; 119°51'50"E), 9 February 2011, P. Bell and S. Catomore leg.; 7 females (WAM C51848) in absolute alcohol, borehole W105 (23°19'37"S; 119°51'50"E), 13 April 2012, P. Bell and S. Catomore leg.; 3 females (WAM C51849) in absolute alcohol, borehole W105 (23°19'37"S; 119°51'50"E), 10 February 2012, S. Catomore and N. Coen leg.; 2 females (WAM C51850) in absolute alcohol, borehole W116 (23°14'47"S; 119°54'25"E), 8 February 2011, P. Bell and S. Catomore leg.; 3 females (WAM C51851) in absolute alcohol, borehole W116 (23°14'47"S; 119°54'25"E), 22 November 2009, P. Bell and G. Perina leg.; 5 females (WAM C51852) in absolute alcohol, borehole W116 (23°14'47"S; 119°54'25"E), 21 April 2010, P. Bell and G. Perina leg.; 8 females and 1 male (WAM C51853) in absolute alcohol, borehole W116 (23°14'47"S; 119°54'25"E), 8 February 2012, S. Catomore and N. Coen leg.; 6 females and 3 males (WAM C51854) in absolute alcohol, borehole W116 (23°14'47"S; 119°54'25"E), 11 April 2012, P. Bell and S. Catomore leg.; 1 female (WAM C51855) in absolute alcohol, borehole W79D (23°19'42"S; 119°50'39"E), 22 November 2009, P. Bell and G. Perina leg.; 3 females (WAM C51856) in absolute alcohol, borehole W79D (23°19'42"S; 119°50'39"E), 12 April 2012, P. Bell and S. Catomore leg.; 2 males (WAM C51857) in absolute alcohol, borehole WP126NRE (23°15'01"S; 119°53'42"E), 21 November 2009, P. Bell and G. Perina leg.

Description. *Female*. Body (Fig. 5A) cylindrical, 450–495 μm (mean 471 μm; n = 6) long (measured from tip of rostrum to posterior margin of caudal rami) and 95–105 μm (mean 103 μm; n = 6) wide (at first free pedigerous somite). Prosome composed of cephalothorax and 3 free pedigerous somites. Urosome comprised of fifth pedigerous somite, genital double-somite, and 3 free abdominal somites. Components of genital double-somite (Fig. 5A, B, C) not fused dorsally but completely fused ventrally, with elliptical integumental window laterally, row of small spinules immediately posterior to each integumental window, and row of large spinules and frill of minute spinules encircling posterior margin; genital field with large median copulatory pore, chitinized copulatory duct leading anteriorly to pair of lobate seminal receptacles, and median genital pore covered by operculiform leg 6. First free abdominal somite with anteroventral pair of oval integumental windows and row of unequal spinules and frill of minute spinules ringing posterior border. Second free abdominal somite with row of subequal spinules and frill of minute spinules encircling posterior edge. Anal somite (Fig. 5B, D, E) with anterior and posterior row of spinules on ventral surface, several rows of spinules on lateral surface, and spinules along posterior margin of anal operculum.

Caudal ramus (Fig. 5B, D, E) about 1.5 times longer than wide, bearing 7 setae. Spinules present at insertion point of setae III, VI and VII. Setae IV and V spinulate, with proximal breaking planes; other setae naked. Seta VII basally tri-articulate.

Rostrum (Fig. 5F) about 1.25 times longer than wide, not demarcated at base, with rounded apex and 2 dorsal sensilla.

Antennule (Fig. 6A) similar to that of *N. knotti* sp. n.

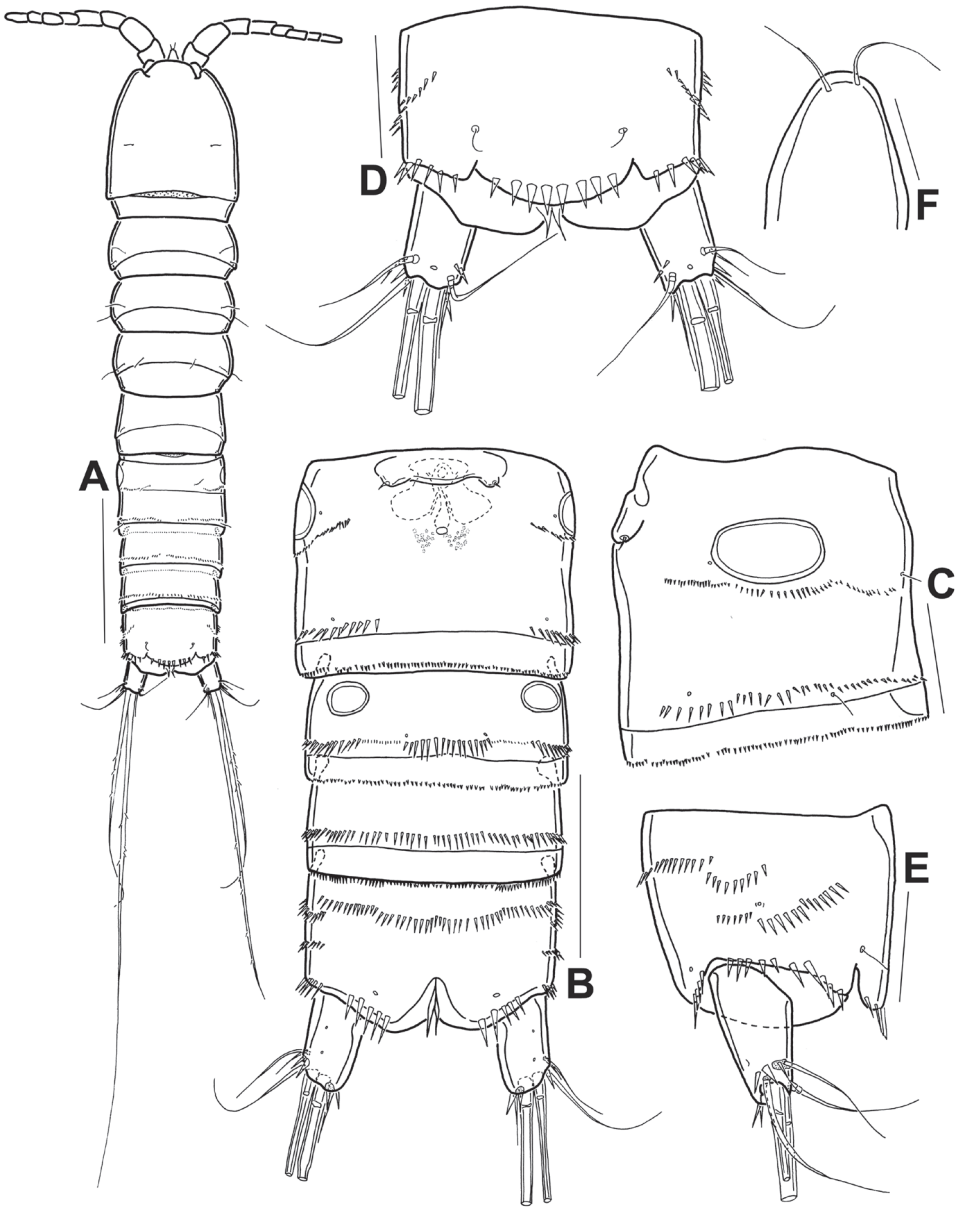

Figure 5. *Nitocrella karanovici* sp. n., adult female: **A** habitus, dorsal **B** urosomites 2–5 and caudal rami, ventral **C** genital double-somite, lateral **D** anal somite and caudal rami, dorsal **E** anal somite and left caudal ramus, lateral **F** rostrum, dorsal. Scale bars: **A** 100 μm; **B** 50 μm **C, D, E** 25 μm; **F** 5 μm.

Antenna (Fig. 6B), comprising coxa, basis, 1-segmented exopod, and 2-segmented endopod. Coxa naked and unarmed. Basis with 2 long spinules on inner margin and short row of minute spinules on anterior surface. Exopod armed with 3 pinnate

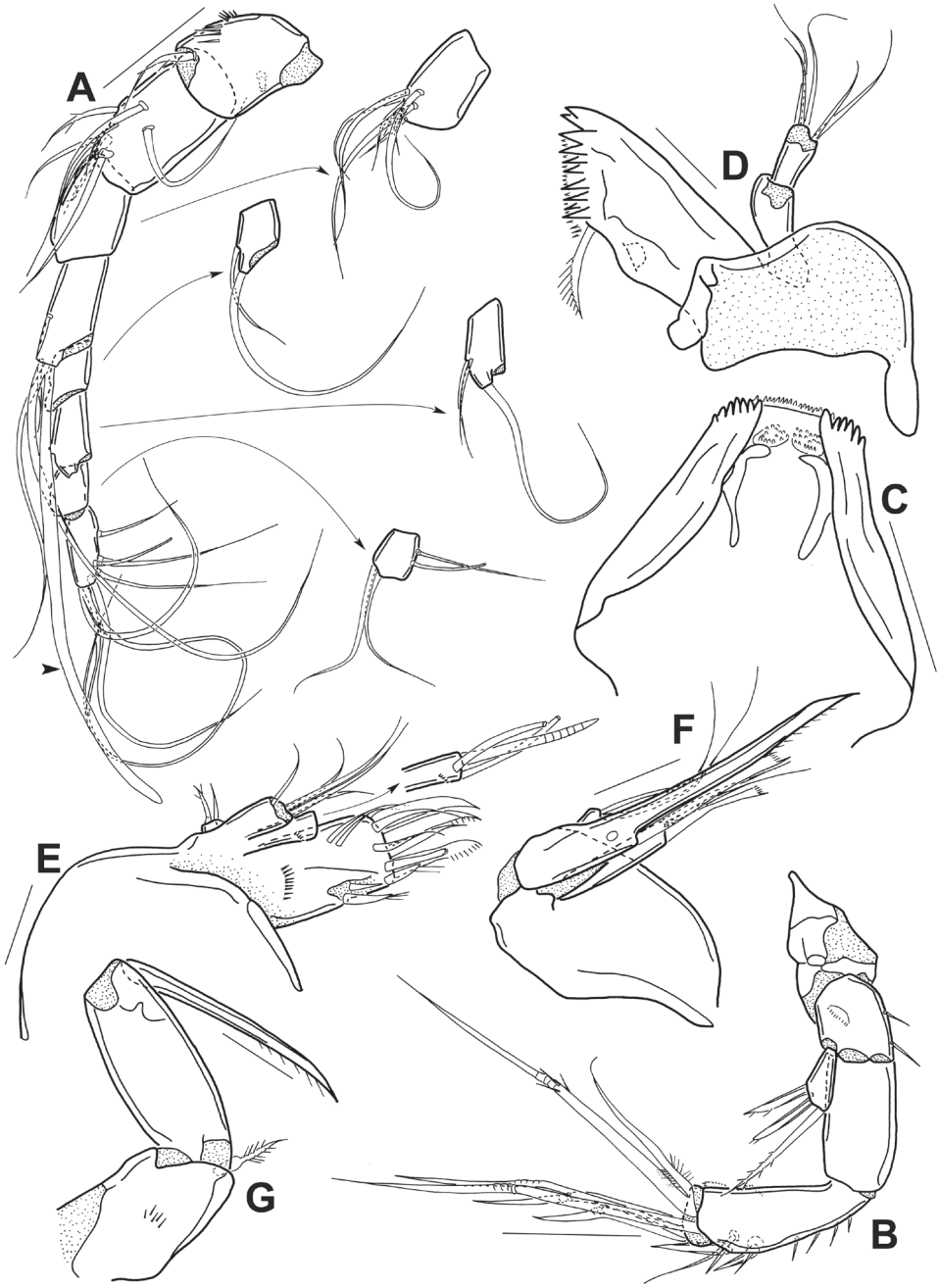

Figure 6. *Nitocrella karanovici* sp. n., adult female: **A** right antennule with segments 3, 5, 6 and 7 shown separately and aesthetasc indicated by arrowhead, ventral **B** right antenna, anterior **C** labrum, posterior **D** left mandible, posterior **E** left maxillule, anterior **F** left maxilla, anterior **G** right maxilliped, posterior. Scale bars: **A** 50 μm; **B, C** 20 μm; **D, E, F, G** 10 μm.

setae (2 highly chitinized). Proximal endopodal segment naked and unarmed. Distal endopodal segment slightly longer than proximal endopodal segment; ornamented with row of spinules on proximal half of inner margin, 2 distolateral hyaline frills, and 2 distomedial spinules; armed distomedially with 2 naked unequal spines plus 1 long naked seta and apically with 1 pilose and 5 geniculate setae (lateralmost geniculate seta with 2 spinules at mid-point and fused basally with pilose seta).

Labrum (Fig. 6C) as in *N. knotti* sp. n., except shorter and with smaller denticles along distal margin.

Mandible (Fig. 6D) similar to that of *N. knotti* sp. n.

Maxillule (Fig. 6E) similar to that of *N. knotti* sp. n.

Maxilla (Fig. 6F) as in *N. knotti* sp. n., except with naked syncoxa.

Maxilliped (Fig. 6G) similar to that of *N. knotti* sp. n., except with only 1 row of spinules on syncoxa and no proximal seta on endopod.

Legs 1–4 biramous (Fig. 7A, B, C, D); leg 1 with trimerous rami; legs 2–4 with trimerous exopod and bimerous endopod. Armature on rami of legs 1 to 4 as follows (Roman numerals = spines; Arabic numerals = setae):

	Coxa	Basis	Exopod	Endopod
Leg 1	0-0	I-I	I-0; I-I; III,2,0	0-I; 0-0; 0,I+2,0
Leg 2	0-0	I-0	I-0; I-I; II,2,1	0-I; 0,I+1,0
Leg 3	0-0	1-0	I-0; I-I; II,2,1	0-I; 0,I+1,I
Leg 4	0-0	1-0	I-0; I-I; II,2,1+I	0-I; 0,I+1,0

Leg 1 (Fig. 7A) intercoxal sclerite naked and concave on posterior margin. Coxa with 1 row of spinules on anterior surface and another row of spinules on posterolateral surface. Basis with row of long spinules at insertion of each ramus and 3 additional large spinules proximal to inner spine; both spines with subapical flagellum. Exopodal segments with large spinules along outer margin and on outer distal corner; middle segment also with fine spinules along inner margin. Endopodal segments with large spinules on outer margin and fine spinules along inner margin. Both setae on terminal exopodal segment and 1 of 3 setae on distal endopodal segment geniculate.

Leg 2 (Fig. 7B) intercoxal sclerite naked and posteriorly bilobate. Coxa with 1 row of minute spinules on posterolateral surface. Basis with row of spinules at insertion of each ramus and several fine spinules (only 1 depicted) on inner margin; outer spine with subapical flagellum. Exopod ornamented as in leg 1, except with additional spinulated frill on inner distal corner of proximal and middle segments. First two exopodal segments protruded on outer distal corner. Both endopodal segments with spinules along outer margin.

Leg 3 (Fig. 7C) similar to leg 2, except with outer seta (instead of spine) on basis and 3 elements on distal endopodal segment.

Leg 4 (Fig. 7D) similar to leg 3, except with much smaller spinules at insertion of endopod, 6 elements on distal exopodal segment (of which inner distal seta is longer

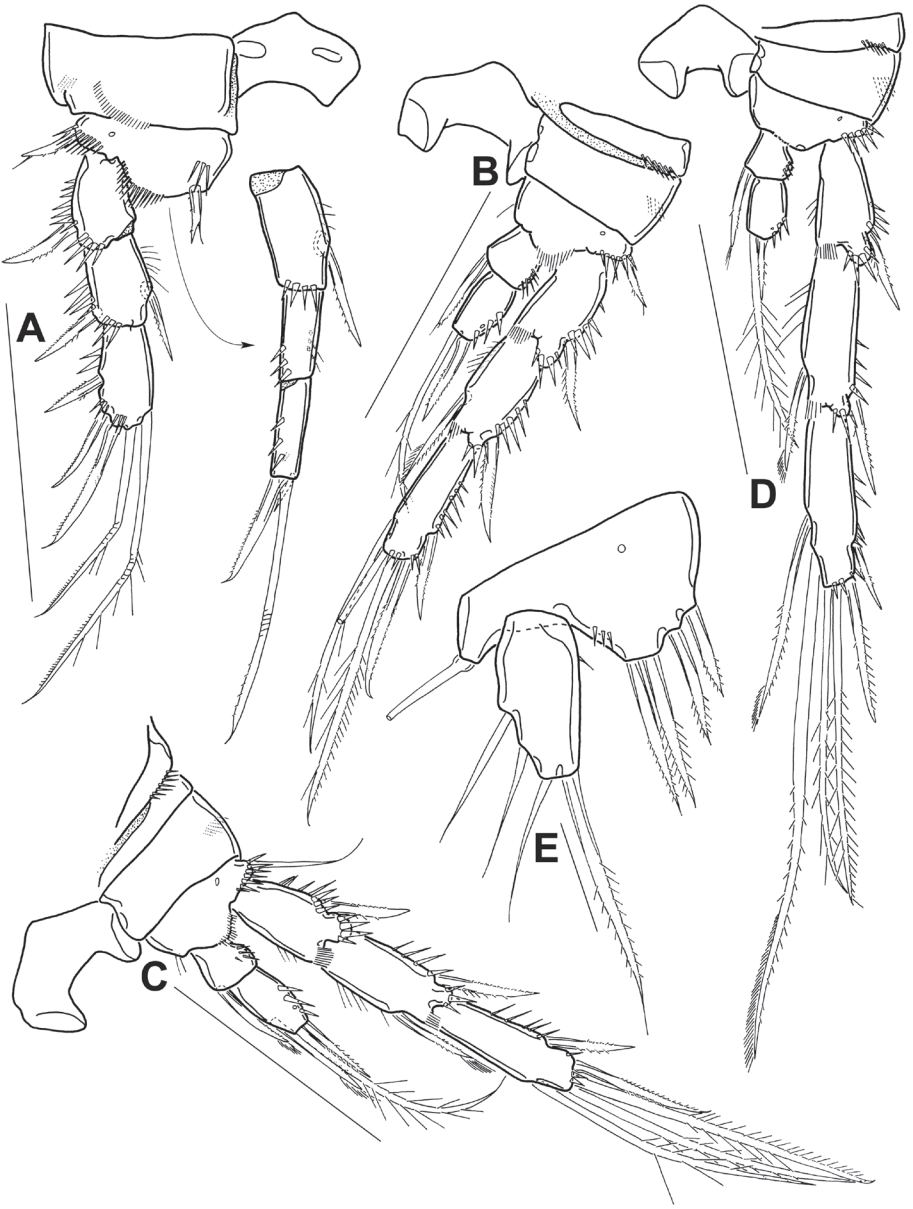

Figure 7. *Nitocrella karanovici* sp. n., adult female: **A** right leg 1 with endopod disarticulated from basis, anterior **B** left leg 2, anterior **C** left leg 3, anterior **D** left leg 4 (note: outer seta on basis is broken off), anterior **E** right leg 5, ventral. Scale bars: **A, B, C, D** 50 μm; **E** 10 μm.

and ornamented with tightly packed spinules on inner margin of apex), and 2 elements on distal endopodal segment.

Leg 5 (Fig. 7E) biramous. Basoendopod with long outer basal seta plus median pore, short row of spinules laterally and 4 distal spinulated setae on endopodal lobe.

Exopod 1-segmented, about twice as long as wide, with spinules along inner margin and 4 setae (3 naked; 1 spinulated).

Leg 6 (Fig. 5B) represented by genital operculum covering genital pore, and armed with 1 minute naked seta on distolateral borders.

Male. Body length (measured from tip of rostrum to posterior margin of caudal rami) 400–440 μm (mean 417 μm; n = 7); body width 90–95 μm (mean 91 μm; n = 7). Prosome composed of cephalothorax and 3 free pedigerous somites. Urosome comprised of fifth pedigerous somite, genital somite and 4 free postgenital somites. Genital somite (Fig. 8A) wider than long, with elliptical integumental window laterally and row of small spinules immediately posterior to each integumental window. First postgenital somite (Fig. 8A) with ventral pair of oval integumental windows, posterior row of large spinules, and frill of minute spinules along posterior border. Second and third postgenital somites (Fig. 8A) posteriorly with row of spinules and frill of minute spinules. Anal somite (Fig. 8A) ornamented as in female. Caudal ramus (Fig. 8A) about 2 times as long as wide; armed and ornamented as in female.

Antennule (Fig. 8B) 10-segmented, haplocerate, with geniculation between segments 7 and 8. Armature as follows: 1, 10, 8, 2, 7 + ae, 2, 3, 4, 4, and 8. Short spinulate seta(e) with flagellate tip present on segments 5–7. Aesthetasc and adjacent apical seta on segment 5 basally fused forming acrothek. One (of 3) and 3 (of 4) elements on segments 7 and 8, respectively, modified as digitate spines. Two apical setae on segment 10 basally fused.

Inner spine on basis of leg 1 (Fig. 8C) modified as is typical for members of Ameiridae.

Leg 5 (Fig. 8D) biramous, with basoendopods fused medially. Basoendopod with outer basal seta and median pore and 2 apical spinulated elements on endopodal lobe. Exopod 1-segmented, about 1.3 times as long as wide, with 5 setae (3 naked; 2 spinulate).

Leg 6 (Fig. 8A) asymmetrical, with right side modified as operculum and left side basally fused to somite; each side armed with 2 unequal distolateral setae.

Variability. One paratype female with discontinuous row of spinules along posteroventral margin of anal somite (Fig. 5B), but row is continuous in other paratype specimens. One dissected paratype female and 1 intact paratype male with 4 elements on terminal exopodal segment of leg 1 (Fig. 8E). One dissected and 1 intact paratype males with 3 elements on distal endopodal segment of leg 2 (Fig. 9C). One dissected paratype female with 4 elements on terminal exopodal segment of leg 3 (Fig. 8F). One dissected paratype female and 1 intact paratype male with 2 elements on distal endopodal segment of leg 3 (Fig. 8G). One dissected and 3 intact paratype females with longer inner distal spine on distal endopodal segment of leg 3 (Fig. 8H). One dissected and 3 intact paratype females plus 3 intact paratype males with 3 elements on distal endopodal segment of leg 4 (Fig. 8I). One intact paratype female with 1 element on distal endopodal segment of leg 4 (Fig. 8J). Two dissected and 1 intact paratype females with 3 (Fig. 9A) or 2 elements (Fig. 9B) on basoendopod of leg 5. Five intact paratype males with 1 (Fig. 9D) or no elements

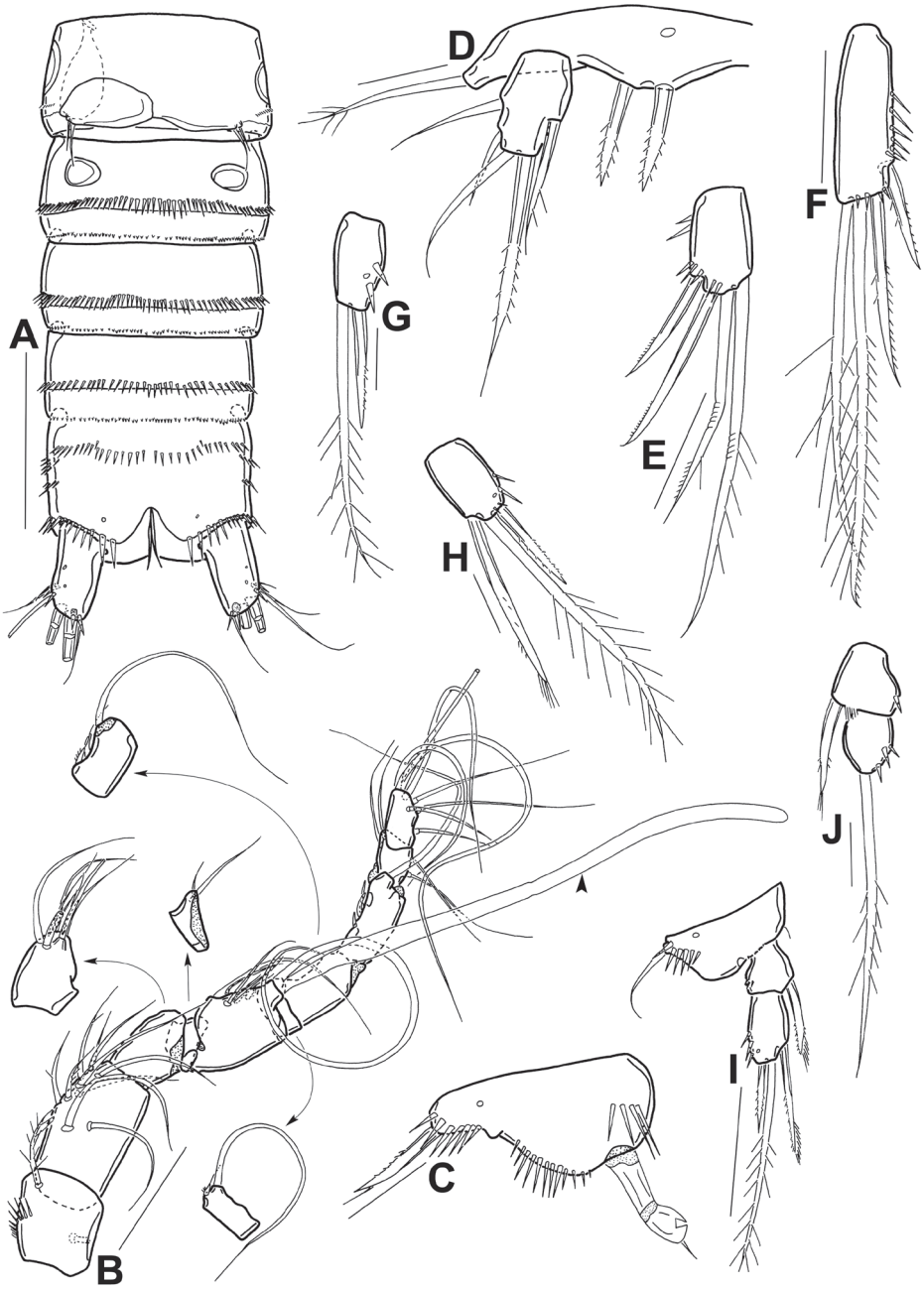

Figure 8. *Nitocrella karanovici* sp. n., adult male (**A, B, C, D**) and adult female (**E, F, G, H, I, J**): **A** urosomites 2–6 and caudal rami, ventral **B** left antennule with segments 3, 4, 6 and 7 shown separately and aesthetasc indicated by arrowhead, ventral **C** right leg 1 basis, anterior **D** right leg 5, ventral **E** terminal exopodal segment of right leg 1, anterior **F** terminal exopodal segment of left leg 3, anterior **G** terminal endopodal segment of left leg 3, anterior **H** same, anterior **I** basis and endopod of right leg 4, anterior **J** endopod of left leg 4, anterior. Scale bars: **A** 50 μm; **B, I** 25 μm; **C, D, G, H, J** 10 μm; **E, F** 20 μm.

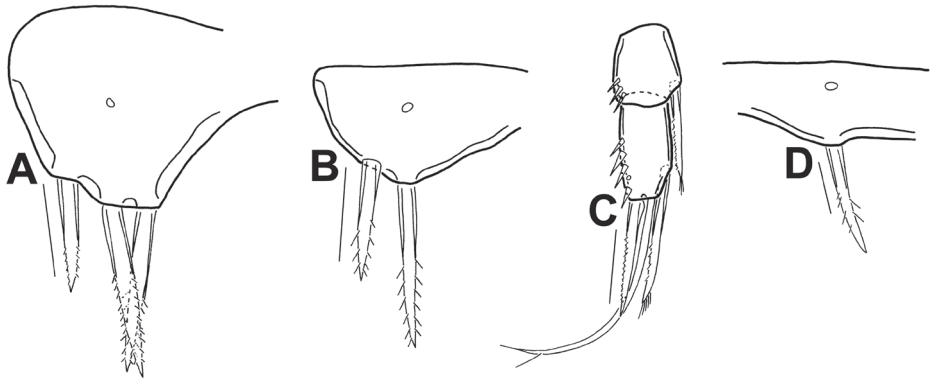

Figure 9. *Nitocrella karanovici* sp. n., adult female (**A, B**) and adult male (**C, D**): **A** basoendopod of left leg 5, ventral **B** same, ventral **C** endopod of right leg 2, anterior **D** basoendopod of left leg 5, ventral. Scale bars: **A, B, C** 10 μm; **D** 5 μm.

(not drawn) on basoendopod of leg 5. One intact paratype male with 3 setae on leg 6 (not drawn).

Etymology. This species is named for Dr. Tomislav Karanovic, in recognition of his extensive taxonomic research on subterranean copepods of Australia.

Differential diagnosis. *Nitocrella karanovici* sp. n. also belongs to the "*vasconica*"-group as it possesses the distinctive six armature elements on the distal exopodal segment of leg 4. Of the other 20 species in this group, *N. karanovici* sp. n. shares five armature elements on the distal exopodal segment of leg 1 with only *N. dussarti* Chappuis & Rouch, 1959 and *N. gracilis* Chappuis, 1955. *Nitocrella karanovici* sp. n. can be easily distinguished from *N. dussarti* by having three armature elements (instead of four) on the distal endopodal segment of leg 3 and four setae (instead of three) on both the exopod and basoendopodal lobe of leg 5 in the female, and from *N. gracilis* by having a short outer spine and long inner seta (rather than two subequal setae) on the distal endopodal segment of leg 2, two spines and one seta (instead of one spine and two setae) on the distal endopodal segment of leg 3, and four setae (instead of 3) on the basoendopodal lobe of leg 5 in the female.

Discussion

This study is of biogeographic interest in providing the first documentation of the genus *Nitocrella* from the Pilbara and Great Sandy Desert regions of northwestern Australia. Previously in Australia, *Nitocrella* was known only from three species in the northern Yilgarn region (Karanovic 2004) (Fig. 1A). Karanovic (2006) recognized that the subterranean copepod fauna is strikingly dissimilar, particularly at the genus level, between the neighboring Pilbara and Yilgarn regions (of which the Murchison region forms a part). Prior to this study, only *Schizopera* G. O. Sars, 1905 and *Nitocrellopsis* Galassi,

De Laurentiis & Dole-Olivier, 1999 were known from both regions. Indeed, the Pilbara region has stronger copepod faunal connections to the Kimberley region in Western Australia, and to northern Queensland, than to the northern Yilgarn region (Karanovic 2006, Karanovic and Hancock 2009). This study brings the number of copepod genera now known to be shared between the Pilbara and northern Yilgarn to three, although the addition of this third copepod genus makes little difference to further understanding of the perplexing taxonomic dissimilarities between these two adjacent stygo-regions.

Karanovic (2010) noted that the species of *Nitocrellopsis* reported from the two regions are only remotely related to each other. The same trend also appears to be the case for the species of *Nitocrella*. For example, although all five species of *Nitocrella* collected from Western Australia belong to the "*vasconica*"-group, they differ from each other in many respects (see Table 3). *Nitocrella karanovici* sp. n. and *N. knotti* sp. n. both have the genital and first abdominal somites fused ventrally, but they differ in the number and position of the integumental windows, the armature of the antennal exopod, and the armature of legs 1 to 5. *Nitocrella obesa* and *N. trajani* share the same number of armature elements on legs 1, 2 and 4, but they differ in the body ornamentation and armature of legs 3 and 5. *Nitocrella absentia* Karanovic, 2004 and *N. karanovici* sp. n. have the same

Table 3. Comparison of morphological characters between female *Nitocrella* species reported from Western Australia.

Character	*N. absentia*	*N. obesa*	*N. trajani*	*N. knotti* sp. n.	*N. karanovici* sp. n.
Genital and first abdominal somites fused ventrally	No	No	No	Yes	Yes
Integumental window on body	Absent	Absent	Absent	1 present on 2nd and 3rd prosomites and on 3rd urosomite	1 pair present on 2nd and 3rd urosomites
Spinules along posterior margin of anal operculum	Present	Absent	Present	Present	Present
No. of setae on antennal exopod	3	3	3	2	3
No. of armature elements on distal exopodal segment of leg 1	4	4	4	4	5
Spinules on distolateral lobes of intercoxal sclerite of leg 2	Absent	Present	Absent	Present	Absent
No. of armature elements on distal exopodal segment of legs 2 and 3	5	4	4	4	5
No. of armature elements on distal endopodal segment of leg 2	4	3	3	4	2
No. of armature elements on distal endopodal segment of leg 3	4	3	4	4	3
Outer spine on middle exopodal segment of leg 4	Absent	Present	Present	Present	Present
No. of armature elements on distal endopodal segment of leg 4	4	3	3	3	2
No. of armature elements on basoendopodal lobe of leg 5	4	1	4	3	4

armature on the exopod of legs 2 and 3 and on the basoendopod of leg 5, but they differ in the number and ornamentation of the urosomites and armature of leg 4.

The collection records for both *Nitocrella karanovici* sp. n. and *N. knotti* sp. n. strongly suggest that each species is a short-range endemic (SRE), with their recorded distribution ranges being much less than the SRE thresholds of 10 000 km^2 nominated by Harvey (2002), or 1 000 km^2 recommended by Eberhard et al. (2009) for Pilbara stygofauna. Both collection localities have been subjected to multiple monitoring surveys over some 15 years. Out of 30 boreholes monitored in eight surveys at Telfer, *Nitocrella* sp. has only been recorded from two closely adjacent boreholes (HB405 and HB406) (Bennelongia 2010). Other stygofaunal taxa collected from borehole HB405 included copepods, ostracods, and paramelitid amphipods. The aquifer type, groundwater depth, and physicochemistry in the Telfer boreholes were not available to this study.

Out of more than 40 boreholes monitored over numerous surveys at Ethel Gorge, *N. karanovici* sp. n. has only been recorded from eight boreholes that span a linear range of less than 14 km (Figure 1B). Twenty-three other stygofauna species were collected from the eight boreholes sampled during the four surveys (2009 to 2012) in Ethel Gorge which detected *N. karanovici* sp. n., including: harpacticoid (3 species) and cyclopoid (4) copepods, candonid (9) and limnocytherid (1) ostracods, paramelitid amphipods (3), tainisopid isopods (1), parabathynellid syncarids (1), and naidid (1) and phreodrilid (2) oligochaetes (Subterranean Ecology 2014). In three boreholes (EEX917, T399, W105), *N. karanovici* sp. n. occurred sympatrically with another ameirid, *Archinitocrella newmanensis* Karanovic, 2006. Both these taxa share a similar habitus and while *A. newmanensis* was often very abundant in samples *N. karanovici* sp. n. was represented by only a few individuals. For these reasons *N. karanovici* sp. n. may have missed detection during earlier monitoring surveys.

Eighty-four species of stygofauna have been recorded from the Ethel Gorge aquifer and adjacent groundwaters in the Newman area, thus ranking it as one of the richest localized groundwater fauna assemblages in Australia, and indeed globally (Subterranean Ecology 2013, 2014, Halse et al. 2014). At least 45 species, including *N. karanovici* sp. n., are considered to be stygobionts (obligate groundwater species) because they possess morphological specializations to subterranean life and are not known from surface waters. Around 40 of the stygobiont species have to date only been recorded from the Ethel Gorge aquifer or adjacent groundwaters in the Newman area (Subterranean Ecology 2013). The Ethel Gorge aquifer stygobiont community is therefore a local hotspot within a regional hotspot. The centre of species richness and abundance for this stygobiont community is concentrated in the shallow alluvial and calcrete aquifer around Ethel Gorge where the Fortescue River flows through the Ophthalmia Range. It is effectively a subsurface gorge where the bedrock shallows and the watertable lies generally less than ten metres below ground level (BHP undated). The distribution range of *N. karanovici* sp. n. more or less coincides with this core centre of stygobiont community richness which extends upstream of the gorge for approximately 2 km and downstream for approximately 6 km (Bennelongia 2015) (Fig. 1B). The groundwater quality in the shallow aquifer is predominantly fresh with measured salinities in most

boreholes less than 1500 mg/L but with some boreholes recording salinities up to around 5,000 mg/L (Subterranean Ecology 2014). The highest salinity groundwater that *N. karanovici* sp. n. was collected from was around 2400 mg/L.

The Ethel Gorge aquifer stygobiont community is listed in Western Australia as an endangered Threatened Ecological Community (TEC) by the Department of Parks and Wildlife (DPaW 2014). The Environmental Protection Authority (EPA 2016) has identified potential impacts on stygofauna habitat and species within the Ethel Gorge TEC from mine dewatering groundwater drawdown and changes in water quality due to the discharge of surplus water into Ophthalmia Dam. As a consequence of its proximity to these potential threatening processes the Ethel Gorge aquifer has received greater sustained survey effort (mostly in the form of environmental compliance monitoring) and taxonomic scrutiny (including molecular genetic studies) than any other groundwater system in Australia. For environmental impact assessment (EIA) and environmental compliance monitoring it is pertinent to note that even considering the intensive biannual field sampling and specimen identification efforts over more than 15 years, new species (including *N. karanovici* sp. n.) continue to be detected from boreholes that have been sampled many times previously (Subterranean Ecology 2012, 2013). While this finding is entirely consistent with intensively studied groundwater systems elsewhere in the world (e.g. Culver and Pipan 2009, Dole-Olivier et al. 2015, Brancelj et al. 2016), this facet of subterranean biology is not widely recognised in EIA for mine projects in Australia.

Acknowledgements

The field surveys at Ethel Gorge were funded by BHP Billiton Iron Ore, and the surveys at Telfer by Newcrest Mining. Professor Pierre Horwitz kindly provided the specimens from Telfer, which were collected by Joe Mifsud. Giulia Perina, Peter Bell, Natasha Coen, and Steven Catomore (Subterranean Ecology Pty Ltd) collected the copepod samples at Ethel Gorge; Giulia also assisted with drafting the maps. The taxonomic descriptions were funded by Subterranean Ecology Pty Ltd. Helpful comments received from two reveiwers were appreciated and improved the manuscript.

References

Bennelongia (2010) Monitoring Program: Taxonomic alignment of stygofauna species. Prepared for Newcrest Mining Limited: Telfer Gold Mine, Report 2010/97.

Bennelongia (2015) Strategic Environmental Assessment: Description of Regional Subterranean Fauna. Prepared for BHP Billiton Iron Ore, September 2015, Report 2015/202.

BHP Billiton (undated) Strategy, Development and Planning – SEA Hydrology – Ecohydrological Change Assessment, 129 pp. http://www.bhpbilliton.com/~/media/bhp/documents/society/regulatory/_ironore/westernaustraliaironore/strategicenvironmentalassess-

ment_centralpilbara_state/appendices/160316_ironore_waio_pilbarastrategicassessment_state_appendix7.pdf?la=en [15 August 2016]

Brancelj A, Žibrat U, Jamnik B (2016) Differences between groundwater fauna in shallow and in deep intergranular aquifers as an indication of different characteristics of habitats and hydraulic connections. Journal of Limnology 75: 248–261. doi: 10.4081/jlimnol.2016.1294

Borutzky EV (1972) Copepoda Harpacticoida from subterranean water of the shore of Issyk–kul and southern Kisilkum. Trudy zoologicheskogo Instituta Akademie Nauk SSSR 51: 98–119. [In Russian]

Boxshall GA, Defaye D (2008) Global diversity of copepods (Crustacea: Copepoda) in freshwater. Hydrobiologia 595: 195–207. doi: 10.1007/978-1-4020-8259-7_21

Boxshall GA, Halsey SH (2004) An introduction to copepod diversity. The Ray Society, London, 966 pp.

Chappuis PA (1937) Subterrane Harpacticoiden aus Nord-Spanien. Buletinul Societatii de Stiinte din Cluj 8: 556–571.

Chappuis PA (1955) Notes sur les copépodes. 18. Nouveaux harpacticoïdes des Pyrénées. 19. Harpacticoïdes cavernicoles de Grèce. 20. Copépodes harpacticoïdes des Iles du Pacifique. Notes Biospéologiques 10: 89–101.

Chappuis PA, Rouch R (1959) Deux nouvelles copépodes cavernicoles des Pyrénées. Annales de Spéléologie 14: 213–218.

Cottarelli V, Bruno MC (1993) Harpacticoida (Crustacea, Copepoda) from subterranean waters of Bue Marino cave, Sardinia, and St. Barthélémy cave, Corsica, and description of three new species. International Journal of Speleology 22: 97–119. doi: 10.5038/1827-806X.22.1.3

Culver DC, Pipan T (2009) The biology of caves and other subterranean habitats. Oxford University Press, Oxford, 254 pp.

Dole-Olivier M-J, Galassi DMP, Fiers F, Malard F, Martin P, Martin D, Marmonier P (2015) Biodiversity in mountain groundwater: the Mercantour National Park (France) as a European hotspot. Zoosystema 37: 529–550. doi: 10.13140/RG.2.1.1723.2480

DPaW (2014) Priority Ecological Communities for Western Australia, Version 21, 4 May 2014, Species and Communities Branch, Department of Parks and Wildlife, Perth.

Eberhard SM, Halse SA, Scanlon MD, Cocking JS, Barron HJ (2005) Assessment and conservation of aquatic life in the subsurface of the Pilbara region, Western Australia. In: Gibert J (Ed.) World Subterranean Biodiversity. Proceedings of an International Symposium, 8–10 December 2004, Villeurbanne, France. University Claude Bernard of Lyon 1, PASCALIS European Research Programme, Lyon, 61–68.

Eberhard SM, Halse SA, Williams M, Scanlon MD, Cocking JS, Barron HJ (2009) Exploring the relationship between sampling efficiency and short range endemism for groundwater fauna in the Pilbara region, Western Australia. Freshwater Biology 54: 885–901. doi: 10.1111/j.1365-2427.2007.01863.x

EPA (2016) Eastern Ridge Iron Ore Proposal – Extension to Orebodies 24, 25 and 32 Report and recommendations of the Environmental Protection Authority, Report 1571, July 2016.

Guzik MT, Austin A, Cooper S, Harvey M, Humphreys W, Bradford T, Eberhard SM, King R, Leys R, Muirhead K, Tomlinson M (2011) Is the Australian subterranean fauna uniquely diverse? Invertebrate Systematics 24: 407–418. doi: 10.1071/IS10038

Halse SA, Scanlon MD, Cocking JS, Barron HJ, Richardson JB, Eberhard SM (2014) Pilbara stygofauna: deep groundwater of an arid landscape contains globally significant radiation of biodiversity. Records of the Western Australian Museum, Supplement 78: 443–483. doi: 10.18195/issn.0313-122x.78(2).2014.443-483

Harvey M (2002) Short-range endemism among the Australian fauna: some examples from non-marine environments. Invertebrate Systematics 16: 555–570. doi: 10.1071/IS02009

Harvey MS, Rix M, Framenau VW, Hamilton Z, Johnson MS, Teale R, Humphreys G, Humphreys WF (2011) Protecting the innocent: studying short-range endemic taxa enhances conservation outcomes. Invertebrate Systematics 25: 1–10. doi: 10.1071/IS11011

Humes AG, Gooding RU (1964) A method for studying the external anatomy of copepods. Crustaceana 6: 238–240.

Humphreys WF (2008) Rising from Down Under: developments in subterranean biodiversity in Australia from a groundwater perspective. Invertebrate Systematics 22: 85–101. doi: 10.1071/IS07016

Humphreys W (2012) Diversity patterns in Australia. In: White WB, Culver DC (Eds) Encyclopedia of Caves (2nd edn). Elsevier, Amsterdam, 203–219. doi: 10.1016/b978-0-12-383832-2.00029-3

Huys R, Boxshall GA (1991) Copepod evolution. The Ray Society, London, 468 pp.

Karanovic T (2004) Subterranean Copepoda from arid Western Australia. Crustaceana Monographs 3: 1–366.

Karanovic T (2006) Subterranean copepods (Crustacea, Copepoda) from the Pilbara region in Western Australia. Records of the Western Australian Museum, Supplement 70: 1–239.

Karanovic T (2010) First record of the harpacticoid genus *Nitocrellopsis* (Copepoda, Ameiridae) in Australia, with descriptions of three new species. Annales de Limnologie 46: 249–280. doi: 10.1051/limn/2010021

Karanovic T, Hancock P (2009) On the diagnostic characters of the genus *Stygonitocrella* (Copepoda, Harpacticoida), with descriptions of seven new species from Australian subterranean waters. Zootaxa 2324: 1–85. doi: 10.11646/zootaxa.2267.1.1

Karanovic T, Eberhard SM, Perina G, Callan S (2013) Two new subterranean ameirids (Crustacea: Copepoda: Harpacticoida) expose weaknesses in the conservation of short-range endemics threatened by mining developments in Western Australia. Invertebrate Systematics 27: 540–566. doi: 10.1071/IS12084

Karanovic T, Eberhard SM, Cooper S, Guzik M (2014) Morphological and molecular study of the genus *Nitokra* (Crustacea, Copepoda, Harpacticoida) in a small palaeochannel in Western Australia. Organisms Diversity & Evolution 15: 65–99. doi: 10.1007/s13127-014-0193-3

Karanovic T, Pesce GL (2002) Copepods from ground waters of Western Australia, VII. *Nitokra humphreysi* sp. nov. (Crustacea: Copepoda: Harpacticoida). Hydrobiologia 470: 5–12. doi: 10.1023/A:1015694015451

Lee W, Huys R (2002) A new genus of groundwater Ameiridae (Copepoda, Harpacticoida) from boreholes in Western Australia and the artificial status of *Stygonitocrella* Petkovski, 1976. Bulletin of the Natural History Museum, London (Zoology Series) 68: 39–50. doi: 10.1017/S0968047002000055

Löffler H (1959) Beitrage zur Kenntnis der Iranischen Binnengewasser. 1. Der Niriz-See und sein Einzugsgebiet. Internationale Revue der gesamten Hydrobiologie und Hydrographie 44: 227–276. doi: 10.1002/iroh.19590440112

Miura Y (1962) Three new harpacticoid copepods from the subterranean waters of Shikoku in Japan. Japanese Journal of Zoology 13: 267–274.

Pesce GL (1980) Two new species of phreatic harpacticoids from Iran (Crustacea: Copepoda). Bijdragen tot de Dierkunde 50: 364–368.

Petkovski TK (1976) Drei neue *Nitocrella*-Arten von Kuba, zugleich eine Revision des Genus *Nitocrella* Chappuis (s. restr.) (Crustacea, Copepoda, Ameiridae). Acta Musei Macedonici Scientarium Naturalium 15: 1–26.

Rouch R (1964) Notes sur les harpacticides. 1. Une nouvelle *Elaphoidella* de l'Ariège. 2. Description des males de *Nitocrella gracilis* et *Nitocrella elegans*. Annales de Spéléologie 19: 525–531.

Shen CJ, Tai AY (1973) Preliminary analysis of the characteristics of the harpacticoid copepod fauna of China and descriptions of some new species. Acta Zoologica Sinica 18: 365–384. [in Chinese with English translation of diagnoses of new taxa]

Štěrba O (1973) Die neuen Harpacticidenarten der Gattung *Nitocrella* (Crustacea, Copepoda) aus Afghanistan. Zoologischer Anzeiger 190: 333–342.

Subterranean Ecology (2012) BHP Billiton Iron Ore Orebody 23/25 Stygofauna Monitoring Annual Report 2011. Report No 2011/01, Subterranean Ecology Pty Ltd, Stirling, 68 pp.

Subterranean Ecology (2013) Ethel Gorge Aquifer Threatened Ecological Community – Consolidated Taxonomy. Prepared for BHP Billiton Iron Ore, December 2013, 96 pp.

Subterranean Ecology (2014) Orebody 23/24/25 and Jimblebar Stygofauna Monitoring 2013–2014. Report No 2014/05, Subterranean Ecology Pty Ltd, Stirling, 47 pp.

Tang D, Knott B (2009) Freshwater cyclopoids and harpacticoids (Crustacea: Copepoda) from the Gnangara Mound region of Western Australia. Zootaxa 2029: 1–70.

Zeidler W (1989) Crustacea. In: Zeidler W, Ponder WF (Eds) Natural History of Dalhousie Springs. South Australian Museum, Adelaide, 79–87.