

Feeding in the dark: are stonefly nymphs good indicators of reference conditions for cave streams? A case study using gut content analysis in Brazilian quartzite caves

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ABSTRACT

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Benthic macroinvertebrates are considered important bioindicators of water quality, and their distribution is influenced by several factors, including food availability. This feeding is an essential factor for all living beings and determines aspects of temporal and spatial population dynamics and relations between individuals and their respective habitats. The present work aimed to analyze the diet of nymphs of the order Plecoptera associated with quartzite caves located in southern Minas Gerais state, Brazil. The stomach contents of 79 specimens were analyzed, and many were empty (40.5 %). Among the nymphs with stomach contents, the most frequent content was fine particulate organic matter. The high amount of individuals with empty content may indicate a low tolerance of these organisms to subterranean environments.

Key words: subterranean, feeding, Plecoptera, food resources, benthos, bioindicators

RESUMO

Alimentando na escuridão: ninfas de Plecoptera são boas indicadoras de riachos de caverna em condições de referência? Um estudo de caso utilizando análises de conteúdo estomacal em cavernas quartzíticas Brasileiras

Macroinvertebrados bentônicos são considerados importantes indicadores de qualidade de água, sendo sua distribuição influenciada por fatores como alimentação. Essa preferência é um fator essencial para seres vivos e determina aspectos da dinâmica populacional temporal e espacial. O trabalho teve como objetivo analisar a dieta de ninfas de Plecopteras associadas a cavernas quartzíticas no sul do estado de Minas Gerais. Foi analisado o conteúdo estomacal de 79 espécimes e muitos estavam vazios (40,5 %). Entre as ninfas que possuem conteúdo estomacal, o mais presente foi a matéria orgânica particulada fina. A grande quantidade de indivíduos com o estômago vazio pode indicar baixa tolerância desses organismos a ambientes subterrâneos.

Palavras chave: *Subterrâneo, alimentação, Plecoptera, recursos alimentares, bentos, bioindicadores*

INTRODUCTION

Knowledge of the diversity patterns of water quality bioindicator groups is especially relevant when predicting the effects of global changes on biodiversity (Brito *et al.*, 2018). Among benthic macroinvertebrates, three bioindicator groups stand out: Ephemeroptera, Plecoptera and Trichoptera (EPT). Such organisms are sensitive to environmental changes, relatively easy to identify, have a high number of taxa and are well distributed in tropical low-order streams (Martins *et al.*, 2017; Siegloch *et al.*, 2017). EPT are considered good bioindicators of water quality and therefore are used in the development of Biological indices and metrics to accurately measure the preservation state of rivers and streams (Couceiro *et al.*, 2012). Furthermore, the extrapolation of a given metric developed in one region to another region usually requires adaptations that take into account the physical and geological features of the evaluated regions (Silveira *et al.*, 2005; Couceiro *et al.*, 2012). Thus, before assigning poor habitat quality to low-order streams that do not have such groups of organisms, it is necessary to ensure that these organisms are present in the “reference condition”. Here, we define “reference condition” as the “minimally disturbed condition” (MDC), described by Stoddard *et al.* (2006) as “the biological state at stream sites that show only slight signs of human disturbances”.

Plecoptera are generally associated with reference conditions, and their distribution is influenced by food, the physical and chemical conditions of water and other factors (Ferreira *et al.*, 2014). These attributes of the group facilitate ecological measurements of the environmental conditions using data on the occurrence and frequency of organisms (Iliopoulou-Georgudaki *et al.*, 2003) as well as feeding data. Many of these organisms are considered predators (Gallo *et al.*, 2010; López-Rodríguez *et al.*, 2012), but they also act as important shredders and are among the main primary consumers in streams with riparian vegetation (Carvalho & Uieda, 2009). In addition, many of these species may also serve as prey for other larger aquatic organisms (Loureiro *et al.*, 2015).

Although the exclusive occurrence of Plecoptera in caves is rare (Tierno de Figueroa & López-Rodríguez, 2010), such organisms can easily access allogenic cave streams. These streams consist of those that run on the surface and then enter the cave environment without sinkholes in their course (Miller, 1996); thus, they are highly connected with the epigeal (surface) environment. However, once these organisms enter the cave environment, they are not necessarily able to establish viable populations due to limitations, mainly in terms of food availability in the subterranean environment. The oligotrophy is a common condition for first-order streams (Fisher & Likens, 1973). However, in subterranean systems, the photosynthetic production is lacking, and leaf and woody debris inputs are greatly reduced (Culver, 1982; Graening & Brown, 2003). Such reduction on food supplies may determine an accidental occurrence of mayflies in caves.

We start from the hypothesis that the occurrence of Plecoptera at low frequencies in the subterranean environment does not necessarily mean that allogenic streams have low environmental quality. The aim of this study was then to evaluate whether stonefly nymphs are good indicators of cave streams in reference conditions. To verify whether plecopterans are suitable for MDC subterranean streams, an analysis was performed of the stomach contents of immature specimens collected in three streams associated with quartzitic caves. The occurrence of a high number of organisms with an empty stomach would be an indicator of a low affinity with the study environment.

MATERIALS AND METHODS

Study area

Three quartzite caves with allogenic drainage were sampled: Mandembe Cave (S 21° 32' 3810" W 44° 47' 5730"), Serra Grande Cave (S 21° 33' 3350" W 44° 49' 1070") and Toca Cave (S 21° 28' 2400" W 44° 40' 0200"), which are all located in the southern region of the state of Minas Gerais, Brazil. Although anthropogenic activity occurs in the regions of the municipalities listed above

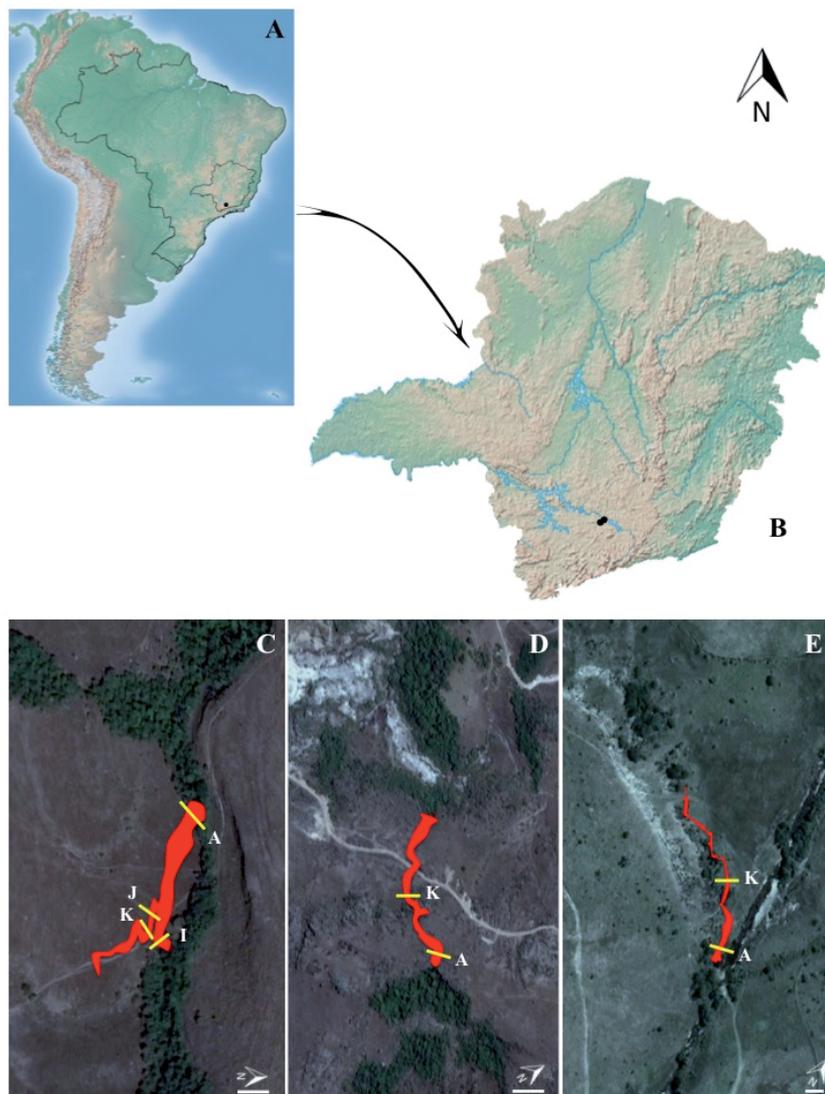


Figure 1. Geographic location of the studied caves; transect A represents the most downstream sampling and K the upstream one. A) South America with Brazil and Minas Gerais state in detail. B) Zoom in the state of Minas Gerais showing the geographic location of the caves in the Paraná river basin. C) Representation of the Mandembe Cave map; bar scale = 20 m. D) Representation of the Serra Grande Cave map; bar scale = 50 m. E) Representation of the Toca Cave map; bar scale = 20 m. *Localização geográfica das cavernas estudadas, o transecto A representa o mais à jusante e o K o mais a montante.* A) América do Sul com o Brasil e Minas Gerais em detalhe. B) Zoom no estado de Minas Gerais mostrando a localização geográfica das cavernas na bacia do rio Paraná. C) Representação do mapa da caverna Mandembe; barra de escala = 20 m. D) Representação do mapa da caverna Serra Grande; barra de escala = 50 m. E) Representação do mapa da Gruta da Toca; barra de escala = 20 m.

(livestock and mining), the riparian forests appear to be intact both upstream and downstream of the caves; therefore, these streams can be considered to present a MDC (Fig. 1). During each sampling event, several physical-chemical parameters were recorded (Table 1).

Invertebrate sampling and identification

In each cave, sampling was performed along a 100-meter stretch of the stream divided in 11 transects. In each transect, three samples were collected using a 400-cm² *Surber* sampler. Since

Table 1. Physical-chemical characteristics of the three sampled caves during the sampling period. The downstream sampling transect represents the entrance area of the cave, the lowest transect. The medium cave stretch represents the medium transect going forward up the cave stream. The upstream transect represents the last collection transect up the cave stream and the most distant transect from the entrance area. *Características físico-químicas das três cavidades amostradas durante o período de amostragem. O transecto de amostragem à jusante representa a área de entrada da caverna, o transecto mais baixo. O trecho médio da caverna representa o transecto médio subindo o riacho da caverna. O transecto a montante representa o último transecto de coleta no córrego da caverna e o transecto mais distante da área de entrada.*

			Temperature (°C)	pH	Conductivity (µs/cm)	O ₂ (mg/L)	Turbidity (NTU)
Mandembe Cave	Down stream	Mean	18.09	4.94	0.01	5.34	0.25
		SD	2.10	0.62	0	0.41	0.28
		Min	15.21	3.53	0	4.73	0.00
		Max	20.81	5.61	0.01	5.89	0.80
	Medium cave stretch	Mean	18.35	4.98	0	6.67	0.90
		SD	2.31	0.58	0	3.02	1.93
		Min	15.04	3.74	0	4.35	0.00
		Max	21.30	5.74	0.01	15.52	6.62
	Up stream	Mean	21.78	5.02	0	4.74	1.21
		SD	0.72	0.58	0	0.91	2.29
		Min	20.86	3.69	0	3.43	0.00
		Max	22.60	5.75	0	6.95	7.40
Serra Grande Cave	Down stream	Mean	17.90	4.93	0.01	6.08	1.66
		SD	2.07	0.34	0.01	3.80	1.91
		Min	14.21	4.29	0	3.91	0.00
		Max	19.92	5.41	0.02	17.31	6.00
	Medium cave stretch	Mean	18.26	5.18	0	5.18	1.30
		SD	1.86	0.27	0	0.86	1.49
		Min	14.95	4.74	0	4.12	0.00
		Max	19.80	5.51	0	6.68	4.20
	Up stream	Mean	18.49	5.29	0	5.11	1.47
		SD	1.53	0.22	0	0.62	1.76
		Min	16.10	4.74	0	4.02	0.00
		Max	20.00	5.51	0.01	6.03	4.60
Gruta da Toca Cave	Down stream	Mean	20.58	4.70	0.01	5.04	2.88
		SD	2.76	0.40	0	0.58	2.69
		Min	17.20	4.04	0.01	4.02	0.00
		Max	27.75	5.37	0.02	6.11	7.50
	Medium cave stretch	Mean	21.14	4.39	0.01	5.16	2.62
		SD	2.39	1.19	0	0.80	2.77
		Min	18.96	0.95	0	3.93	0.00
		Max	27.75	5.47	0.02	6.51	8.70
	Up stream	Mean	21.39	4.78	0.01	4.57	3.73
		SD	2.29	0.32	0.02	0.47	3.51
		Min	19.50	4.33	0	3.71	0.00
		Max	27.75	5.49	0.06	5.29	12.10

the Mandembe Cave main stream has less than 100 meters in length, two transect were positioned in a lateral tributary. The sampling was repeated monthly, for a total of 11 sampling events in each cave. From the samples obtained in the field, Plecoptera were separated from the other species of benthic organisms using a stereomicroscope (ZEISS - STEMI 2000). The organisms were then identified to the genus level. The identification was performed using the taxonomic key (Mugnai *et al.*, 2010) and a stereomicroscope (ZEISS - STEMI 2000).

Gut content analysis

To assess the gut contents, each specimen had its abdomen dissected and gut opened with the aid of a scalpel and entomological pins. All gut contents were removed, mounted on slides and coverslips, and then analyzed under a microscope. The gut contents were classified into four categories: *i*) FPOM (fine particulate organic matter, represented by fragmented pieces smaller than 1 mm); *ii*) CPOM (coarse particulate organic matter, represented by fragmented pieces larger than 1 mm); *iii*) PT (plant tissue); and *iiii*) AT (animal tissue) according to the categorization proposed by Azzouz & Sánchez-Ortega (2000). Plant tissue and animal tissue were distinguished by cell type under the microscope. The gut contents were analyzed for the frequency of occurrence of each food item (independently of their size), which is the most efficient method of qualitatively analyzing diets (Azzouz & Sánchez-Ortega, 2000). In this way the same individual could present more than one category of food item classification. Based on the gut content analysis of all specimens, a list was prepared with the various types of food with records of the presence or absence of food items in the gut of each specimen analyzed. After analyzing all specimens, the proportion of organisms containing one or more items of a given type of food was calculated as the frequency of occurrence of that type of food.

We investigated the existence of differences in the stoneflies nutritional quality performing a Mann-Whitney test, using `wilcox.test` function, in the stats package in R program (R Core Team, 2018). Finally, we represented the stoneflies gut

contents spatially, according to each cave collecting transect.

RESULTS

A total of 85 Plecoptera individuals were found in the caves, which represent approximately 4.3 % of the total EPT found in the cave streams under study (Table 2). The gut contents were analyzed for 79 Plecoptera individuals. For the others six specimens dissection and gut opening was not possible due to their very small size or due to a high degree of body tissues damage. Among the total of Plecoptera, 42 were collected in the Mandembe Cave, 14 in the Serra Grande Cave, and 29 in the Toca Cave. Of this total, 34 belonged to the family Perlidae and were distributed in the genera *Kempnyia*, *Anacroneuria* and *Macrogynoplax*, and 51 belonged to the family Gripopterygidae and were distributed in the genera *Tupiperla*, *Gripopteryx* and *Paragripopteryx*.

None of the specimens analyzed had CPOM or PT in their gut. Of all the analyzed individuals, the gut was empty in 32 individuals, which represented approximately 40.5 % of the total analyzed individuals. Among the individuals with an empty gut, 20 belonged to the family Gripopterygidae, and only 12 belonged to the family Perlidae. The most widely distributed food item among the analyzed organisms was FPOM (Table 3, Fig. 2), which was present in approximately 59.4 % of the analyzed organisms. For all individuals in which animal tissue was found, there was also FPOM. Among the food items found, it was possible to identify organisms of the families Simuliidae and Chironomidae, both belonging to the order Diptera.

The stoneflies' body size was not related to their nutritional quality content. The spatial distribution of the stoneflies in the three caves did not show a gradient from the entrance to the deeper caves' regions (Fig. 3). In Mandembe Cave, it was possible to observe an increase in the FPOM gut content in the transects close to cave entrance. The Serra Grande Cave stoneflies showed a bigger amount of FPOM in the transect close to entrances, and such content became less abundant downstream. Finally, in Toca Cave it was possible to see an increase of

Table 2. Abundance of the EPT genera in each cave. *Abundância de gêneros de EPT em cada caverna.*

	Gruta da Toca	Gruta Mandembe	Gruta Serra Grande
EPHEMEROPTERA			
Baetidae			
<i>Adebrotus</i>		4	
<i>Americabaetis</i>		8	5
<i>Apobaetis</i>		1	1
<i>Baetodes</i>		7	
<i>Cloeodes</i>	12	18	12
<i>Paracloeodes</i>	1	11	
<i>Tupiara</i>		1	
<i>Waltzoyphius</i>	4	56	
<i>Zelusia</i>	1	78	14
Caenidae			
<i>Caenis</i>	1		
Leptohyphidae			
<i>Tricorythopsis</i>		4	
Leptophlebiidae			
<i>Askola</i>	1	286	80
<i>Hagenulopsis</i>		188	36
<i>Massartella</i>		23	
<i>Simothraulopsis</i>		338	11
PLECOPTERA			
Gripopterygidae			
<i>Gripopteryx</i>	1	3	
<i>Paragripopteryx</i>	3	5	
<i>Tupiperla</i>	14	23	2
Perlidae			
<i>Anacroneuria</i>	9	1	5
<i>Kempnyia</i>	2	10	6
<i>Macrogynoplax</i>			1
TRICHOPTERA			
Anomalopsychidae			
<i>Cotulma</i>			2
Calamoceratidae			
<i>Phylloicus</i>	43	23	11

Cont.

Table 2. (cont.)

Ecnomidae			
<i>Austrotinodes</i>	1		
Helichopsychidae			
<i>Helicopsyche</i>		26	3
Hydrobiosidae			
<i>Atopsyche</i>		8	3
Hydrophilidae			
<i>Derallus</i>			1
Hydropsychidae			
<i>Leptonema</i>	16		1
<i>Macrostemum</i>		6	
<i>Smicridea</i>	16	1	1
Hydroptilidae			
<i>Alisotrichia</i>	14	86	10
<i>Leucotrichia</i>		1	
<i>Neotrichia</i>	2	211	34
<i>Oxyethira</i>		8	
Leptoceridae			
<i>Grumichella</i>		10	
<i>Nectopsyche</i>	1	23	10
<i>Nectopsyche</i>		1	
<i>Notalina</i>		13	1
<i>Oecetis</i>	3	14	13
<i>Triplectides</i>	7	3	
Odontoceridae			
<i>Barypenthus</i>		6	
<i>Marilia</i>		1	
Odontoceridae			
<i>Barypenthus</i>		1	
<i>Marilia</i>		1	
Polycentropodidae			
<i>Cyrnellus</i>			
<i>Polycentropus</i>	5	18	21
TOTAL	157	1526	284

Table 3. Food item frequency for each stonefly nymph genus. FPOM: fine particulate organic matter; AT: animal tissue; PT: plant tissue; CPOM: coarse particulate organic matter. Since the same individual presented more than one food item classification, the sum of all item categories exceeded 100%. *Frequência de itens alimentares para cada gênero de ninfa de Plecoptera. FPOM: matéria orgânica particulada fina; AT: tecido animal; PT: tecido vegetal; CPOM: matéria orgânica particulada grossa. Como ocorreu mais de uma classificação de item alimentar para mesmo indivíduo, a soma de todas as categorias ultrapassou 100%.*

Family	Genera	Empty	FPOM	AT	PT	CPOM
Gripopterygidae	<i>Tupiperla</i>	16	20	0	0	0
	<i>Gripopteryx</i>	2	2	0	0	0
	<i>Paragripopteryx</i>	2	6	0	0	0
Perlidae	<i>Kempniya</i>	4	12	7	0	0
	<i>Anacroneuria</i>	8	6	0	0	0
	<i>Macrogynoplax</i>	0	1	0	0	0
Total / percentage		32 (40.5%)	47 (59.4%)	7 (8.8%)	0	0

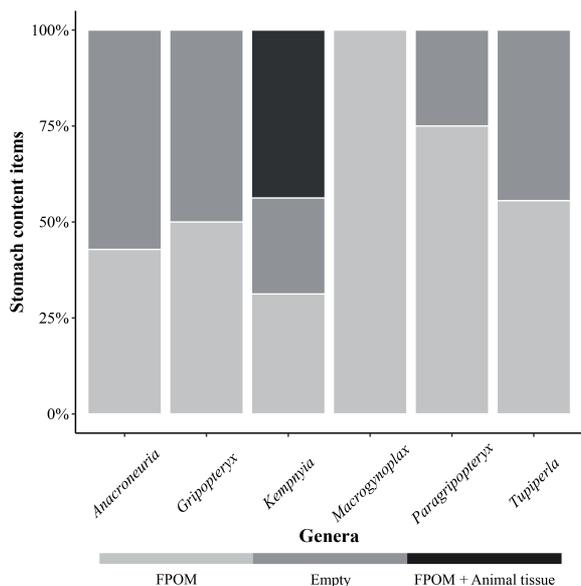


Figure 2. Percentage of the food items ingested by each stonefly nymph genus. *Porcentagem dos itens alimentares ingeridos por cada gênero de ninfa de Plecoptera.*

FPOM in the gut content in the last three collecting transects, located downstream in the cave.

DISCUSSION

In this work, only few individuals of Plecoptera were found when compared to other EPT groups

found in the same caves. The proportion of these organisms tends to be much higher in surface aquatic invertebrate communities (Azzouz & Sánchez-Ortega, 2000; Kikuchi & Uieda, 2005; Loureiro *et al.*, 2015). While some Trichoptera and Ephemeroptera are more diverse and tolerant to a wider range of conditions, Plecoptera are found only in waters with a higher quality (Galdean *et al.*, 2000). In general, the fact that caves are environments with limited resources generates a longitudinal colonization gradient, where the entry zones are richer and more abundant in invertebrates compared to more inner areas (Watson, 2010; Gomes *et al.*, 2009). However, this pattern was not observed in the studied caves. Although a low abundance of predatory organisms, such as Perlidae, was expected (Vannote *et al.*, 1980; Watson, 2010), the abundance found was extremely low. The lower richness of cave communities compared to epigeal communities has been widely reported in the literature (Gomes *et al.*, 2009; Prous *et al.*, 2004). The peculiar characteristics of these environments hinder their colonization (Pipan & Culver, 2012); therefore, the aquatic communities in caves often represent subsets of the communities associated with surface streams (Gunn *et al.*, 2000; Watson, 2010).

Although it is considered an oligotrophic environment, the Mandembe Cave had the largest

number of Plecoptera individuals. Most likely, greater colonization by Plecoptera was observed in this cave because it is the smallest cave analyzed in this study and consequently is under a greater influence of the surface environment. The colonization of lotic cave environments often occurs through drift (Kohler, 1985) because the environment presents low food availability and total absence of light, many of these organisms release themselves into the current to escape from caves and find a more favorable environment (Death, 1989). Thus, larger caves favor this behavior, and few individuals actually settle in the caves. In addition, individuals of Plecoptera are sensitive to disturbances, and their occurrence is dependent on the quality of the aquatic environment (Ferreira *et al.*, 2014). Thus, the search for more favorable environments becomes even more evident.

The availability of organic matter within the lotic cave environment is dependent on the influx

of allochthonous resources, which in turn are directly related to the flow regime of the cave stream. Given the slow flow of the streams analyzed here, the influx of organic matter into the caves is low, which severely hinders the feeding of Plecoptera. Therefore, the caves may eventually become transitional environments for Plecoptera in search of an environment with greater food availability. In addition, few individuals were found with food items in their guts, and in a large proportion, their guts were empty.

The fact that the environment is oligotrophic does not necessarily imply a high incidence of organisms with an empty gut. A study conducted in Himalayan high-altitude lagoons, which represent environments that are also considered extremely oligotrophic, did not find as high a proportion of individuals with empty guts, and only approximately 11 % of the evaluated Capniidae (Plecoptera) had empty guts (Boggero *et al.*,

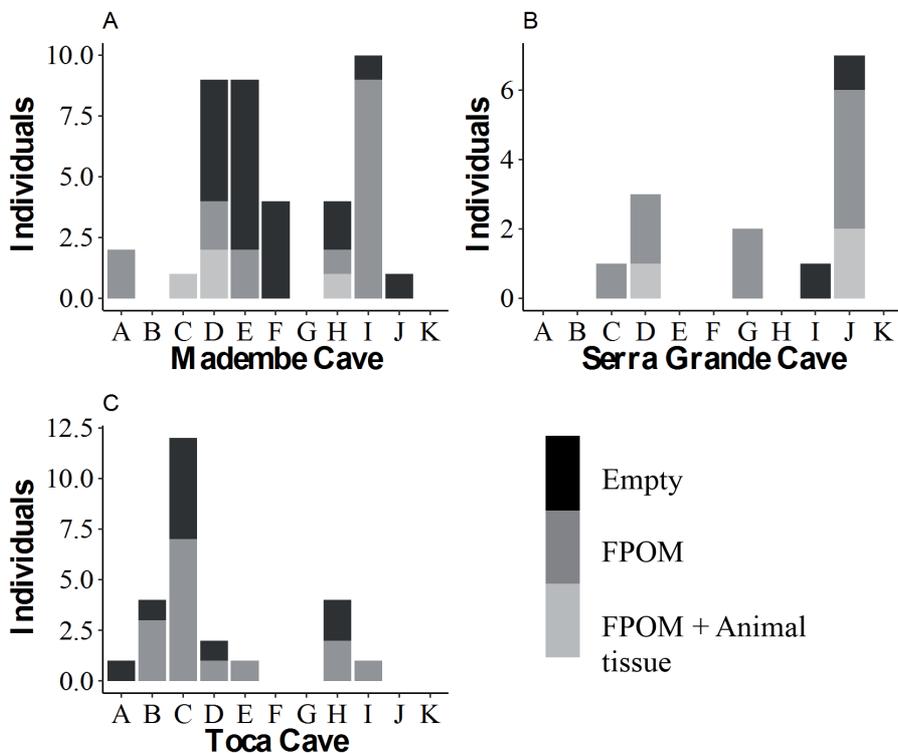


Figure 3. Spatial representation of the stoneflies gut contents analyses found in each cave. Transect A represents the most downstream collection site of each cave and transect K upstream. *Representação espacial do conteúdo estomacal dos plec6pteras encontrados em cada caverna. O ponto A representa o local de coleta mais à jusante de cada caverna e o ponto K o mais a montante.*

2014). The cave stonefly *Protonemura gevi* (Tierno-de-Figueroa & López-Rodríguez, 2010) adapted to the subterranean environment, has also a higher rate of empty guts, of approximately 39 % (López-Rodríguez & Tierno de Figueroa, 2015). Therefore, other factors must have contributed to the high occurrence of organisms without gut contents in the caves analyzed here. Stoneflies are opportunistic, and the food ingested may vary depending on the species developmental stage and whether its preferred food item is in low abundance (Stewart, 1984). The nymphs can even spend periods without feeding, which may occur given the lack of prey or during the larval molting stages. In addition, predators (represented by Perlidae) have periods during which they do not feed either because of a lack of prey availability or during the molting period (Ferreira, 1996; Magni-Darwich *et al.*, 1999). The predation rate of Plecoptera decreases as environmental conditions become more stressful (Thomsom *et al.*, 2002). Among the Perlidae, only individuals of the genus *Kempnyia* had prey in their gut contents, which does not necessarily mean that the organisms have been feeding inside the caves. Given the small length of the caves, the nymphs may have fed at the surface environment before their collection inside caves. Predation occurs via the detection of prey by a combination of mechanical, chemo sensitive and visual structures (Peckarsky, 1982); thus, predation is impaired in an environment with a total absence of light.

Regarding the stoneflies that did feed, most of them had FPOM in the gut contents, confirming that Plecoptera contribute to the fragmentation of the leaf detritus in the streams (Graça, 2001; Loureiro *et al.*, 2015; Azzouz & Sánchez-Ortega, 2000). In contrast, among the cave *P. gevi* specimens, the main food item found was CPOM (López-Rodríguez & Tierno-de-Figueroa, 2015). The adaptation of *P. gevi* to the cave environment probably provides it with differentiated structures that enable more efficient access to food resources in an aphotic and oligotrophic environment. On the other hand, in high-altitude oligotrophic environments, the family Capniidae, which is generally categorized as collector-gatherer and shredder, becomes mainly a collector-gatherer and feeds on FPOM due to the low CPOM availability (Bogge-

ro *et al.*, 2014). Such changes in the proportions of food items are expected in the surface stoneflies that access the caves, and the large proportion of individuals with an empty gut provide a strong indication of the low adaptations of stoneflies to the subterranean environment.

A study on the dietary habits of Gripopterygidae in the Northern Hemisphere revealed that the species from this family are herbivore-detritivores (Yule, 1986). The study also reports a changing in the feeding habits with the increasing nymphal size. Larger species consume a higher quality nutritional diet in terms of protein and calorific content, with larger amounts of filamentous green algae and diatoms in their guts. FPOM is found in all analyzed species, however such item was considered the least nutritionally valuable of the food items (Yule, 1986). Regardless the body size, the most frequently food item found among the stoneflies individuals analyzed in this study was the FPOM. However, it is worth mentioning that filamentous green algae and diatoms are not expected to be found in the stoneflies guts due to the complete darkness of the caves.

The spatial pattern of gut content found in Mandembe Cave clearly represents the oligotrophy of the lateral tributary, which is more isolated from the surface. The Serra Grande Cave was the only one that showed a gradient of reduction of gut content from the entrance towards deeper regions of the cave. This pattern was expected due to the intense gradient found in caves following the light that became totally absent in profound cave regions (Watson, 2010). The smaller amount of FPOM found in the transects closer to the surface in Toca Cave (J and K) occurs probably because those transects are about 300 meters far from the entrance while in Serra Grande cave it is about 50 meters far. The increase in FPOM in the downstream transects (A, B and C) is once again due to a submerged bat guano deposit found between the transects B and C in this cave. Bat guano is an important organic impute resource in caves, which can influence in the aquatic fauna densities (Fenolio *et al.*, 2005). The lack of a similar pattern for the three caves is expected, since subterranean environments are known to be unique (when compared to each other) due to physical (structural), hydrological

and biological distinctions between them (Pellegrini *et al.*, 2018).

Although the existence of the true cave Plecoptera *P. gevi*, the analyzed quartzite caves were not a favorable environment for colonization by this group. The results of this study show that Plecoptera should not be included in biological indices for measuring habitat quality in caves because they have low affinity with the subterranean environment. Nevertheless, further studies covering a larger number of caves are needed to provide a better understanding of this family dynamics in this highly peculiar environment.

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