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Anchialine biodiversity in the Turks and Caicos Islands: New discoveries and current faunal composition


1Smithsonian Institution, National Museum of Natural History, Department of Invertebrate Zoology, P.O. Box 37012, Washington D.C., USA
2Molecular Ecology Group, Water Research Institute, National Research Council of Italy, Largo Tondoli 50, 28922 Verbania Pallanza, Italy
3Natural History Museum, University of Copenhagen, Universitetsparken 15, Copenhagen Ø, Denmark
4Marine Biological Section, Department of Biology, University of Copenhagen, Universitetsparken 4, Copenhagen Ø, Denmark
5Marine Science Center, Northeastern University, Nahant, Massachusetts, USA
6Marine Biology Department, Texas A&M University at Galveston, Galveston, Texas, USA
7Monterey Bay Aquarium Research Institute, Moss Landing, CA, USA
8Professional Technical Diver, Hudson, Florida, USA
9Big Blue Collective, Leeward Highway, Leeward Settlement, Turks and Caicos Islands
10Department of Environment and Coastal Resources, Grand Turk, Turks and Caicos Islands
11Dive Provo, Grace Bay Road, Grace Bay, Turks and Caicos Islands

Abstract: Lying at the southernmost point of the Lucayan Archipelago, the Turks and Caicos Islands are amongst the better studied localities for anchialine cave biodiversity. For nearly five decades, novel invertebrate fauna, comprised primarily of crustaceans, have been collected from these tidally influenced pools – but new findings are always on the horizon. Herein we present new records of crustaceans and annelids from anchialine blue holes and horizontal caves of the Turks and Caicos. These findings include two potentially new species of meiofaunal annelids and a new species of remipede collected from a shallow water cave pool. Our 2019 expedition additionally expands known faunal distributions for several taxa across the Caicos islands, and raises the biodiversity of the region to 35 species, 13 of them considered endemic. This is the first comprehensive faunal list for the anchialine systems in the Caicos Bank.

Keywords: subterranean fauna, anchialine, Polynoidae, Nerillidae, Remipedia


INTRODUCTION

Anchialine ecosystems are characterized as tidal, brackish water bodies with subterranean connections to the sea (Holthuis, 1973). Originally described as tidally influenced pools, cave diving explorations in the late 20th century highlighted that these systems are often associated with extensive subterranean labyrinths (Stock et al., 1986; Iliffe, 2000, 2005). Biological research in anchialine systems continues to yield new and exciting taxa, of uncertain phylogenetic affinities, within classes, orders and families that are often found exclusively in anchialine caves (Iliffe, 1986, 2000). Some of these species are phylogenetically old, presumably colonizing the anchialine realm in the Tertiary (Iliffe, 2005), while others exhibit close affinities to deep sea groups (Gonzalez et al., 2018; Iliffe & Alvarez, 2018) or represent highly modified members of otherwise interstitial lineages (Worsaae et al., 2004, 2019b; Martínez et al., 2016b).

Anchialine systems are known from the Caribbean, Mediterranean, Indo-Pacific and Atlantic islands including Bermuda, Canary Islands, Ascension and even Iceland (Iliffe et al., 1983; Iliffe & Sarbu, 1990; Biernbaum, 1996; Kristjánsson & Suvavársson, 2007; Martínez García et al., 2009; Pérez-Moreno et al., 2016). The most extensive of these systems in terms of density and size of subterranean passages occur

*gonzalezbc@si.edu
§These authors contributed equally
in the Northern Caribbean, particularly in the Yucatán Peninsula (Alvarez et al., 2015), Cuba (Orghidan et al., 1973), Jamaica (Kornicker & Iliffe, 1992) and Hispaniola (Trias et al., 1997) as well as in the adjacent Lucayan Archipelago, which includes the Bahamas and the Turks and Caicos Islands (Palmer, 1985).

The Turks and Caicos Islands (Fig. 1A), and the neighboring islands of the Bahamas, are known for their high number of cave adapted anchialine species, prime for scientific exploration (Botosaneanu, 2000). Anchialine water bodies in this karstic area are primarily accessed through blue holes and horizontal caves (Gregor, 1981), harboring a diverse community of locally endemic, cave-limited species dominated by crustaceans, annelids and other invertebrates (Kornicker & Iliffe, 1985; Suárez-Morales & Iliffe, 1996; Koenemann et al., 2007). The first cave adapted species reported from the Turks and Caicos were the shrimp *Barbouria cubensis* (von Martens, 1872) and *Typhlatya garciai* Chace, 1942, discovered in 1975 from easily accessible pools and karstic windows (Buden & Felder, 1977). However, the majority of the diversity was not discovered until after 1982 once scientific cave divers started exploring the subterranean labyrinths (Bowman et al., 1985; Holsinger & Yager, 1985; Kornicker & Iliffe, 1985). We here provide an overview of these discoveries, which we further update with the results of our “2019 Caicos Caves III Expedition” with the aim of obtaining a comprehensive genetic collection of anchialine fauna across cave systems of the Turks and Caicos Islands. A prospective on the general state of knowledge, as well as on potential conservation issues of these ecosystems, are provided.

**MATERIALS AND METHODS**

**Geological settings of the Turks and Caicos Island**

The Caicos Islands are the emerged part of the Caicos Bank, the southernmost platform in the Lucayan Archipelago. This large shallow water bank is surrounded by waters reaching oceanic depths, with the present-day Caicos Islands being located along the northern and western margins. Strong easterly trade winds and resulting wind-wave agitation have promoted the development of oolitic sands in the shallow water interior of the platform (Dravis & Wanless, 2008). Washed ashore on the islands, these oolitic sands have piled into Holocene and Pleistocene age ridges, oriented parallel to the shoreline and perpendicular to prevailing easterly trade winds. These shallow water carbonates that comprise the bank have been continuously depositing and subsiding since at least the Cretaceous, but possibly as early as the Jurassic (Schlager et al., 1984), and extend deep into the surrounding sea floor (Dietz et al., 1970). The slow subsidence of the bank due to the continually accumulating weight of new carbonates has maintained the top of the bank near sea level. Yet, during Pleistocene periods of sea level regressions, the entire top of the carbonate bank was exposed to atmospheric weathering, producing the characteristic karst terrain. The climate of the islands is tropical and semi-arid with an average temperature of 26°C and annual rainfall of 730 mm. The karst topography of the islands is dominated by dolines (closed depressions) produced by limestone solution or collapse into underlying caves (Guidry et al., 2007). Collapsed caves on land consist of deep circular pits with a talus cone in the center, while occasional large vertical shafts or blue holes lie in shallow waters of the interior bank. Cavernous porosity underlying the...
islands resulted from dissolution along abundant fracture networks, running parallel to the bank margin (Guidry et al., 2007).

History of previous cave expeditions


Sampling collection – Smithsonian

“Caicos Caves III”

Our 2019 Caicos Caves III expedition was sponsored by the Smithsonian Institution with the aim of obtaining a comprehensive genetic collection of anchialine fauna across cave systems in the Turks and Caicos Islands. In order to accomplish this, fauna was unbiasedly collected across all available access points, including caves, ponds and wells with different environmental conditions, such as wide-ranging salinities (brackish to fully haline) and varying light exposure (entrance pools, twilight-, constant dark zones). Specific details pertaining to faunal collections are listed in Supplemental Table and detailed below.

Cave fauna was collected by scientific cave divers using 20 mL glass scintillation vials or 50 mL plastic Falcon tubes. Prior to capture, water-filled vials and tubes were flushed with ambient cave water to prevent osmotic shock. For smaller fauna, plankton nets with removable cod-ends were employed (Iliffe & Alvarez, 2018). Separate cod-ends were used for fresh- and saltwater layers. Upon returning to the surface, samples were placed in lidded cooling boxes until processing. All samples were processed within 24-48 hrs.

Initial processing included photographing and assigning all samples museum voucher numbers. Live specimens were relaxed with an isotonic solution of MgCl₂, then photographed using a mounted Canon EOS 5DSR with dual-slave Speedlite 430EX II strobes. Morphological examinations were done with light microscopy (LM) using Olympus SZX10 and SZX16 microscopes (Olympus, Tokyo, Japan).

Since the primary focus of this expedition was to obtain genomic quality DNA across anchialine fauna, hologenophore vouchers (Pleijel et al., 2008) were prepared to ensure molecular sequences could be tracked back to individual specimens. This semi-destructive approach allows for identification and additional morphological analyses. Morphological vouchers were typically preserved in either 3% glutaraldehyde or 2–4% paraformaldehyde, with the occasional preservation in 96% EtOH when necessitated by downstream protocols. Removal of several taxonomically non-informative structures (pereopods, parapodia, antennae, trunk limbs, etc.) was performed to obtain genomic quality tissue. Dissected parts were placed in alphanumeric tubes preloaded with 150 µL of M2 buffer (AutoGen) and stored at 4°C. Paragenophore vouchers of select individuals were preserved in 96-100% EtOH for DNA analyses and in Trizol or RNAlater for RNA analyses. All paragenophore vouchers were stored at -20°C in the field and transferred to -80°C upon returning to the Smithsonian National Museum of Natural History.

Permissions for specimen collection and export were granted through a scientific research permit (SRP No.: 18-12-01-23) issued by the TCI Department of Environment and Coastal Resources (DECIR) issued to TMI. All specimens are deposited at the Smithsonian National Museum of Natural History, Washington, D.C., USA (USNM 1524194–1524351).

RESULTS

Seven anchialine cave systems yielding significant faunal discoveries are known from the Turks and Caicos Islands: four in Providenciales, one in North Caicos, one in Middle Caicos and one in South Caicos. Detailed descriptions for each cave are provided below; exact cave locations have been withheld per the guidelines of our scientific research permit. Specific faunistic information for each cave can be found in Supplemental Table. From this point forward, we
use the term “stygobite/stygobitic” to refer to those species exclusively known from cave systems, while those species that are only recorded from the Turks and Caicos are referred to as “endemic”.

Caycos caves
Caves in Providenciales Island

Airport Cave is located at the crest of a hill overlooking Providenciales International Airport car park. It is about 2 km inland from the coast near the midpoint of the island of Providenciales at the northwestern corner of the Caicos Bank. The cave entrance is a crescent-shaped fissure extending off the side of a much larger, but debris-choked, sinkhole. A narrow ravine-like passage extends down for 20 m to a tidal anchialine pool lying in total darkness. The maximum water depth is 14 m. A colony of bats roost over the pool and their guano makes up much of the sediment. Based on our most recent visit, this cave should be considered biologically ‘dead’ in terms of macrofauna, as water pollution and hydrogen sulfide render the water anoxic and unsuitable for supporting animal life. Seven stygobitic species had been previously recorded from this locality, four of which are endemic (see Supplemental Table).

Old Blue Hill Cave is 300 m to the northeast of Airport Cave on Providenciales. Two pools on opposite sides of a large collapsed sinkhole are separated by massive breakdown boulders. The largest pool, beneath a 7 m high limestone cliff, opens to sunlight and has several meters of tannic surface water. Below the tannic layer, the water clears and a restricted hole opens into a larger chamber, sloping down and then choked off by a breakdown at 25 m depth. The second pool is sheltered from direct sunlight in a shallow cave and contains clear water. Seven stygobites are known from this locality, four of which are considered endemic (see Supplemental Table).

Snake Cave is located about 1.5 km inland and 1.1 km north of the airport on Providenciales. It consists of a long and narrow, mostly water-filled, fissure forming the margin of a larger collapsed sink. Although parts of the cave pool are dimly illuminated, the back section remains in total darkness. Maximum water depth is 8 m. Due to its remoteness, this cave is rarely visited, with only one stygobite being recorded (see Supplemental Table).

The Hole is a deep sinkhole, located at the crest of Long Bay Hills, a prominent ridgeline on the southwestern side of Providenciales. The cave is a 50 m diameter and 18 m deep sinkhole with vertical walls of soft limestone and 200 m from the nearest open water (Juna Sound on the south coast). Sheer walls surrounding a pool show a close resemblance to cenotes found in northern Yucatan Peninsula, Mexico. This 15 m long by 10 m wide pool at the bottom of the depression is open to daylight. It is about 6 to 8 m deep, but the bottom is completely choked by breakdown and surface debris, such that no human-sized cave passages extends off from it. A total of nine stygobites have been recorded from this cave, having the highest diversity on Providenciales (see Supplemental Table).

Caves in North Caicos Island

Cottage Pond is a submersed sinkhole, located 4 km inland from the north coast of North Caicos Island (Fig. 1B). A shallow, rock-rimmed depression encloses a nearly circular pond, 50 m in diameter. At the surface, murky water limits underwater visibility to about 1–3 m. However, near the center of the pond (20 m depth), a 12 m long horizontal crack opens into a collapsed chamber (70 m in diameter) that reaches water depths of 80 m. A conical debris mound lies directly below the entrance crack (~40 m depth) and extends downward to the bottom of the deep chamber. Evidence of prior subaerial exposure in the form of stalactites and flowstone can be found down to 45 m. Layers of hydrogen sulfide accumulate at around 40 m depth. Currently the best known system in North Caicos, a total of 15 stygobitic (five endemics) are known from Cottage Pond, exhibiting the highest diversity of all known anchialine systems in the Turks and Caicos (see Supplemental Table).

Caves in Middle Caicos Island

Conch Bar Cave, with over 3 km of horizontal passageways, is the largest subaerial cave in the Lucayan Archipelago (Fig. 1C) (Smart et al., 2008). The cave consists of a series of large chambers and irregular passageways along the southern margin of an eolianite ridge. A series of phreatic formed, elliptical shaped passages with domed ceilings run towards the interior of the ridge. Passage ceiling elevations extending up to 22 m above sea level may be related to former mixing zones (haloclines) or water tables. The centrally located main entrance is in a 40 m diameter collapsed depression. From this breakdown entrance chamber, partially flooded passages extend to the east and north. Clear water in these passages is generally about 0.5–1 m deep, although depths upwards of 15 m are present in several areas farther into the cave. Scattered throughout the cave, many isolated pools fluctuate tidally indicating the presence of subterranean connections between them. Conch Bar Cave has the second highest diversity in the Turks and Caicos, with 14 recorded stygobites, eight of which are endemic from the islands (see Supplemental Table).

Caves in South Caicos Island

The Fountain is a tidal spring with brackish water flowing from a narrow crack, being used as the water supply to flood neighboring salt flats. This is the only system known from South Caicos, with only a single recorded stygobite (see Supplemental Table).

Fauna

Based on the review of all published references for the islands, as well as our new results, 35 anchialine species across three phyla have been recorded in the Turks and Caicos. These include three undetermined species from earlier expeditions (see Supplemental Table), plus three new discoveries during the “Caicos Caves III Expedition”. Crustacea comprise most of the records, being represented by 28 species. Among Crustacea, Copepoda is the most numerous group with 12 species (4 Cyclopoida, 8 Calanoida), of which
five are considered stygobitic (see above). Remipedia is the second largest crustacean group with five species, all stygobitic. Ostracoda and Decapoda have three known species each. While the decapods are all stygobitic, only one ostracod is considered to be stygobitic. Amphipoda is represented by two stygobitic species, while only single species are known for Leptostraca, Stygiomysida and Isopoda.

Other phyla represented in the subterranean environments of the Turks and Caicos Islands are Mollusca, with four species, and Annelida, with three species. All the annelids are considered stygobitic, as opposed to the mollusks, which are exclusively epigean species. All the annelids are considered stygobitic, as opposed to the mollusks, which are exclusively epigean taxa, only capable of surviving at cave entrances.

During the “Caicos Caves III” faunal investigations, we recovered three new cave exclusive species, including two annelids belonging to the genera Mesonerilla and Speleonerilla, and a remipede possibly belonging to a new genus.

A detailed overview of the most relevant anchialine species recorded throughout the Turks and Caicos Islands, including newly collected material, is provided below, including their distribution, phylogenetic affinities and some general remarks. These species are selected based on their phylogenetic or ecological relevance (stygobites) within the cave systems. Accidental species, records lacking formal identification or exclusively epigean species known from cave entrances are only mentioned in Supplemental Table.

Phylum Annelida
Suborder Aphroditiformia
Family Polytonidae
Pelagomacellicephala iliffei Pettibone, 1985
Fig. 2A

Material examined. Turks and Caicos Islands, North Caicos Island, Cottage Pond, 9 January 2019, 33 m, 1 specimen (juvenile), USNM 1524287; 10 January 2019, 33-44 m, 3 specimens, USNM 1524314, USNM 1524315, USNM 1524316.

Distribution. Previously only known from Conch Bar Cave, Middle Caicos Island (type locality), but with new collections from Cottage Pond, North Caicos Island.

Remarks. Stygobitic. This is a very rare species that has only been collected twice after its original species description. It is a predator, typically swimming in saline waters of the cave water column. Pelagomacelliccephala iliffei originally was known from a single cave locality in Middle Caicos at shallow depths (1–1.5 m), but this latest expedition expands both its distribution and depth range to include Cottage Pond on North Caicos. Individuals putatively identified as P. iliffei have been collected throughout the Bahamas, including the islands of Exumas, Eleuthera, Cat and Long, although they differ in body size and number of segments; constituting genetically distinct evolutionary entities (Gonzalez et al., 2017).

Pelagomacelliccephala iliffei is closely related to the only other known anchialine cave scale worm, Gesiella jameensis (Hartmann-Schröder, 1974) from La Corona lava tube in Lanzarote, Canary Islands, Spain (Martinez et al., 2016a). Both species are phylogenetically related to members of the subfamily Macelliccephalinae (Gonzalez et al., 2018; Martinez & Gonzalez, 2018), and therefore, putatively have a deep sea ancestry (Gonzalez et al., 2018; Zhang et al., 2018).

Family Nerillidae
Mesonerilla sp.
Fig. 2B

Material examined. Turks and Caicos Islands, Middle Caicos Island, Conch Bar Cave, 4 January 2019, 0.5-2.0 m cave pools, 2 specimens, USNM 1524205, USNM 1524206.

Distribution. Only known from Conch Bar Cave, Middle Caicos Island.

Remarks. Stygobitic. Members of this genus typically inhabit interstitial marine sands (Worsaae, 2014). However, in anchialine caves, related species are found in coarse sand sediments or lapilli patches as well as hovering over fine silty sediment (Worsaae et al., 2009). Interstitial or epibenthic species of Mesonerilla are deposit feeders, collecting biofilms or deposited organic matter with the help of their club-shaped palps and buccal ciliation. The records presented herein are the first for the genus from the Turks and Caicos.

Mesonerilla sp. is characterized by the presence of a mid-ventral terminal prolongation of the pygidium, a feature shared by the marine interstitial species M. katharinae Worsaae, Mikkelsen & Martinez, 2019 and M. peteri Worsaae, Mikkelsen & Martinez, 2019 from the Caribbean; as well as for the anchialine species M. xurxoi Worsaae, Mikkelsen & Martinez, 2019 from Lanzarote and three undescribed species from Bermuda, México and Cuba (Worsaae et al., 2019a).

Speleonerilla sp.
Fig. 2C

Material examined. Turks and Caicos Islands, Middle Caicos Island, Conch Bar Cave, 4 January 2019, 0.5-2.0 m cave pools, 2 specimens, USNM 1524243, USNM 1524244.

Distribution. Only known from Conch Bar Cave, Middle Caicos Island.

Remarks. Stygobitic. All animals were collected from the water column using a plankton net. This genus was originally described from anchialine caves of Bermuda as Longipalpa (Worsaae et al., 2004), but was recently changed to Speleonerilla as the previous name was preoccupied (Worsaae et al., 2019b). Speleonerilla only inhabits anchialine caves, being found anywhere between brackish and fully haline waters in twilight and constant dark zones. Unlike all other Nerillidae, members of Speleonerilla are suspension feeders and live in the water column. This is the first record of the genus in the Turks and Caicos.

Speleonerilla is sister to several interstitial species of the genus Mesonerilla, suggesting that the lineage colonized the water column of anchialine caves.
from marine interstitial environments (Worsaae, 2003). Phylogenetic analyses have found the genus monophyletic, with *S. isa* Worsaae, Gonzalez, Kerbl, et al., 2018, from Lanzarote, branching near a clade containing the Caribbean species (Worsaae et al., 2019a). *Speleonerilla* sp. from the Turks and Caicos resembles other Caribbean species morphologically, but unpublished DNA sequences indicate that it may represent a new species (Worsaae et al. unpublished). Microscopical examinations of, e.g., gonoducts and ciliation patterns are currently being conducted in search of diagnostic morphological features.

The only other species described from this genus is *A. intermedius* Fosshagen & Iliffe 2007, exclusively known from caves in Andros and Exuma islands, Bahamas. Both species of *Azygonectes* typically share their habitat with other copepod species, including cyclopoids and ridgewayiid calanoids, which most likely constitute prey items.

Epacteriscidae entirely consists of stygobitic species (Fosshagen & Iliffe, 2004a), except for *Miheptneria abyssalis* Andronov, 2007, which has been recorded in the northeast Atlantic at 4622 m deep. Therefore, a deep-sea affinity can be attributed to the family.

**Bofuriella spinosa** Fosshagen & Iliffe, 2007

Distribution. Only known from the type locality, Conch Bar Cave, Middle Caicos, Turks and Caicos Island.

Remarks. Stygobitic. Only collected once from the water column in shallow tidal pools in Conch Bar Cave, most of which are open and exposed to low levels of light. These pools frequently accumulate guano from resident bat populations and range from brackish to fully haline, suggesting its tolerance to varying salinities.

Two additional species in the genus are known from the Bahamas: *B. parvorata* Fosshagen & Iliffe 2007 from Cat Island and *B. vorata* Fosshagen, Boxshall & Iliffe 2001 from the Exuma Cays (Fosshagen et al., 2001; Fosshagen & Iliffe, 2007). As with other epacteriscids, all species of *Bofuriella* are raptorial predators.

**Caiconectes antiquus** Fosshagen & Iliffe, 2007

Distribution. The type locality is designated as Cottage Pond, North Caicos Island, Turks and Caicos Islands; however, additional specimens have been collected in Conch Bar Cave on Middle Caicos Island, and Old Blue Hill Cave on Providenciales, Turks and Caicos Islands.

Remarks. Stygobitic. *Caiconectes* is a monotypic genus endemic from Caicos Islands (Fosshagen & Iliffe, 2007), found across a wide range of depths and only from the water column. It may be found in entrance pools, twilight or in constant darkness zones. This monotypic species is rarely observed, but has been collected in several localities throughout the Turks and Caicos. Hydrological conditions at the time of collection show high tolerances to variabilities in salinity, temperature and oxygen. Caiconectes has the appearance of a typical hyperbenthic calanoid with raptorial feeding.

**Erebonectoides macrochaetus** (Fosshagen & Iliffe, 1994)

see Fosshagen & Iliffe (1994); Fosshagen et al. (2001)

Distribution. Only known from two caves. The type locality is The Hole, Providenciales, Turks and Caicos Islands, with additional records from Conch Bar Cave, Middle Caicos, Turks and Caicos Islands.
Remarks. Stygobitic. This rare monotypic genus is endemic from Caicos Islands, where it is only known from shallow areas of two anchialine caves with intermittent light levels. It is morphologically similar to Erebonectes, represented by the Bermudian endemic species E. nesioticus Fosshagen 1985 (Fosshagen & Iliffe, 1985). As other epacteriscids, E. macrochaetus is a raptorial predator on small swimming cave organisms.

**Family Fosshageniidae**


Distribution. Only known from the type locality, Conch Bar Cave, Middle Caicos Island, Turks and Caicos Islands.

Remarks. Stygobitic. This rare species is exclusively known from a single collection in the shallow, open pools of Conch Bar Cave (0.5-1 m depth), which are subject to periods of illumination and variability in salinities.

The genus *Fosshagenia* is known from one additional species, *F. suarezii* Fosshagen & Iliffe 2004a, endemic to Grand Bahama (Bahamas) (Fosshagen & Iliffe, 2004a). The family Fosshageniidae also includes deep-sea species of the genus *Temoropia* (Boxshall & Halsey, 2004).

**Class Malacostraca**

**Order Amphipoda**

**Family Hadziidae**

*Bahadzia stocki* Holsinger, 1985

Fig. 3A

Material Examined. Turks and Caicos Islands, Providenciales, The Hole, 8 January 2019, 4 – 6 m, 9 specimens, USNM 1524279-1524285.

Distribution. This amphipod inhabits several caves on Providenciales, including Airport Cave (type locality), The Hole, Snake Cave and Old Blue Hill Cave, and on Middle Caicos Island from Conch Bar Cave. Additional localities include several Bahamian Islands, including Abaco (Dan’s Cave) and the Exuma Cays – Great Guana Cay (Oven Rock Cave) (Stock & Vermeulen, 1982; van der Ham, 2002).

Remarks. Stygobitic. Specimens were collected in shallow to intermediate depths (~1-10 m) in cave pools where they were found in dimly illuminated to completely dark waters. No obvious morphological differences have been observed between the different cave populations of *S. provo* across the Lucayan Archipelago, but molecular analyses may reveal the presence of cryptic species or evolutionary units as it has been showed in other stygobites with similar distribution patterns (Gonzalez et al., 2017).

The genus *Spelaeonicippe* includes one additional species, *S. buchi* (Andres 1972), endemic to a volcanic cave on Lanzarote, Canary Islands, Spain (Andres, 1975). *Spelaeonicippe provo* and *S. buchi*, along with *Antronicippe serrata* Stock & Iliffe 1990, which is endemic from Grieta de la Caleta de la Torta on Santa Cruz Island, Galapagos (Stock & Iliffe, 1990), belong to the family Pardaliscidae, otherwise entirely consisting of deep-sea species. This suggest a deep sea affinity for species of *Spelaeonicippe*, yet the relationships between deep sea and cave species in these groups have not yet been investigated (Iliffe et al., 1984; Martinez et al., 2016a).

**Order Isopoda**

**Family Cirolanidae**

*Bahalana caicosana* Botosaneanu & Iliffe, 2003

Fig. 3C

Material examined. Turks and Caicos Islands, Middle Caicos Island, Conch Bar Cave, 4 January 2019, 0.5 – 2.0 m cave pools, 1 specimen, USNM 1524211.

Distribution. The type locality is Cottage Pond, North Caicos Island with additional records from Conch Bar Cave, Middle Caicos Island, Turks and Caicos Islands.

Remarks. Stygobitic. The type material of this isopod was collected from 25-46 m depths in Cottage Pond, as opposed to the shallow depths in Conch Bar Cave in which they were found during the Caicos Caves III expedition. The genus *Bahalana* includes five additional species from the Bahamas and Cuba. Bahamian species include *B. abacoana* Botosaneanu

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*R. E. F. Stock & Vermeulen, 1982*

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& Iliffe, 2006 (Abaco), B. cardiopus Notenboom, 1981 (Acklins and Mayaguana), B. exumina Botosaneanu & Iliffe, 2002 (Exumas) and B. geraci Carpenter 1981 (Andros, Abaco, Grand Bahama and San Salvador); while one Cuban species is known, B. boumani Ortiz, Lalana & Pérez 1997 (Ciénaga de Zapata, Matanzas).

The family Agostocaridiidae includes three other cave species: A. acklinsensis Álvarez, Villalobos & Iliffe, 2004 from Acklins Island (Bahamas); A. bozanici Kentsley, 1988 from Yucatán Peninsula (México); and A. zabaleita Mejía-Ortiz, Yañez & López-Mejia, 2017 from Cozumel Island (México) (Kentsley, 1988; Álvarez et al., 2004; Mejía-Ortiz et al., 2017). Species of the family morphologically resemble those in the anchialine family Anchialocarididae, as well as the deep-sea families Alvinocarididae and Mirocarididae (Álvarez et al., 2004; Mejía-Ortiz et al., 2017), suggesting that ancestors of Agostocaris might have colonized anchialine environments from the deep sea.

Family Barbouriidae

Barbouria cubensis (von Martens, 1872)

Material examined. Turks and Caicos Islands, Middle Caicos Island, Conch Bar Cave, 4 & 7 January 2019, 0.5–2.0 m deep cave pools, 3 specimens, USNM 1524202, USNM 1524257, USNM 1524259.

Distribution. Widespread throughout the Bahamas, the Turks and Caicos Islands, Bermuda, Cuba, Cayman Islands, Jamaica and in a few locations along the Caribbean coast of the Yucatán Peninsula, México.

Remarks. Stygobitic. Shrimp were collected from shallow cave pools, where they were found swimming near the guano-rich bottom or amongst the crevices. Pools were in total darkness or dimly illuminated and contained brackish waters. This species is probably a scavenger based on their attraction to baited traps, as well as observations from aquaria (Mejía-Ortíz, 2010; Ditter et al., 2015).

Barbouria cubensis is one of the first described anchialine species, initially reported from Cuba (Barbour, 1945) and afterwards throughout the Caribbean from a wide range of habitats, including open pools and dark cave sections containing brackish to fully haline waters. Despite this broad distribution, animals from geographically distant localities show morphological variation but lack genetic structure based on mitochondrial markers (Ditter et al., 2019), suggesting that they disperse across different island banks. Larval dispersal across distant islands has been also suggested for the Indo-Pacific anchialine shrimp Metabetaus lohena (Russ et al., 2010).

The family Barbouriidae includes B. cubensis, together with the Mexican B. yanezi Mejia, Zarza & López, 2008, four species of the genus Parhippolyte and the deep sea genus Ligur, suggesting a potential deep sea origin for these anchialine shrimp (Hart Jr et al., 1985).

Family Atyidae

Typhlatya garciai Chace, 1942

Material examined. Turks and Caicos Islands, Providenciales, Old Blue Hill Cave, 5 January 2019, 01-04 m, 16 specimens, USNM 1524213, USNM 1524219-1524226. Middle Caicos Island, Conch Bar Cave, 4 January 2019, 0.5-2.0 m cave pools,
10 specimens, USNM 1524197-1524198, USNM 1524201, USNM 1524204, USNM 1524207-1524211.

Distribution. The type locality is Cuba, Holguin Province, Banes; however, T. garciai has been recorded in the Turks and Caicos from the following localities, Conch Bar Cave, Middle Caicos Island, and Old Blue Hill Cave and Snake Cave on Providenciales. From Cuba, localities include Gran Caverna de Santo Tomás (Pinar del Río), cave “Las Cuatrocientas Rozas” in Potrero de Molino and several casimbas (local name for large cracks and crevices amongst the coralline rocks) around Puerto Pesquero (both in Holguin).

Remarks. Stygobitic. These small shrimp are found in waters of salinities ranging from fresh to fully haline. Large populations can literally carpet the bottom at shallow depths of some illuminated cave pools. Interestingly, Botello et al., (2013) found T. garciai to be genetically identical to T. kakuki Alvarez, Iliffe & Villalobos, 2005 from caves on Acklins Island in the Bahamas. Given the slight morphological variations, the underlying genetic diversity appears to be low, and population level genetics are needed to understand relationships between individuals from the known localities.

Order Leptostraca
Family Nebaliidae
Speonebalia cannoni Bowman, Yager & Iliffe, 1985

Material examined. Turks and Caicos Islands, North Caicos Island, Cottage Pond, 6 & 7 January 2019, 22–44 m, 4 specimens, USNM 1524241-1524242, USNM 1524264-1524265.

Distribution. The type locality is Airport Cave with additional records from The Hole, Providenciales, Cottage Pond, North Caicos Island, and from caves in Great Abaco (Bahamas).

Remarks. Stygobitic. Our specimens were observed swimming in the water column of the cave, in total darkness. Specimens were collected from two caves (The Hole and Cottage Pond), but not in Airport Cave due to water pollution. The wide distribution of this species across the Turks and Caicos and the Bahamas needs to be reinvestigated using molecular methods and more detailed morphological studies.

Class Ostracoda
Order Halocyprida
Deeveya spiralis Kornicker & Iliffe, 1985

Material examined. Turks and Caicos Islands, North Caicos Island, Cottage Pond, 7 January 2019, 1 specimen. No museum number assigned.

Distribution. Known from the type locality, The Hole Cave, Providenciales, Turks and Caicos Islands; as well as from Cottage Pond, North Caicos Island.

Remarks Stygobitic. Our specimen was collected using a vial from the water column, where it was seen swimming in total darkness.

Six additional described species make up the genus, all of them from caves in the Bahamas: D. medix Kornicker in Kornicker, Yager & Williams, 1990 from Grand Bahama, Exuma and Abaco; D. bransoni Kornicker & Palmer 1987 and D. hirpex Kornicker in Kornicker, Yager & Williams, 1990 from Andros; D. exleyi Kornicker & Iliffe 1998 from the Exumas; and D. jilae Kornicker & Iliffe 1989 from Eleuthera. Molecular studies are needed to further understand relationships between these species.

Order Stygiomysida
Family Stygiomysidae
Stygiomysis clarkei Bowman, Iliffe & Yager, 1984

Material examined. Turks and Caicos Islands, Middle Caicos Island, Conch Bar Cave, 4 & 7 January 2019, 0.1 – 2.0 m deep cave pools, 6 specimens, USNM 1524195-1524196, USNM 1524214, USNM 1524258, USNM 1524266, USNM 1524267.

Distribution. Known from Conch Bar Cave (type locality), Middle Caicos Island, and Snake Cave, Providenciales. This species is also known from Lucayan Caverns, Grand Bahama Island, Bahamas (Bowman et al., 1984; Pesce & Iliffe, 2002).

Remarks. Stygobitic. These blind, unpigmented crustaceans were found swimming near the guano and mud bottoms of shallow cave pools. Pools were in total darkness or faintly illuminated.

Stygiomysis is a genus with seven described exclusively stygobitic species distributed throughout the Caribbean and Italy. Stygiomysis aemete Wagner, 1992 from Pedernales (Dominican Republic); S. cokei Kallmeyer & Carpenter, 1996 from Quintana Roo and Yucatán (Yucatán Peninsula, México); S. holthuisi (Gordon 1958) from Anguilla, Saint Martin, Puerto Rico, Grand Bahama, Quintana Roo and Yucatán (México); S. ibarrae Ortiz, Lalana & Perez 1996 from Matanzas (Cuba); S. major Bowman, 1976 from Jackson Bay (Jamaica); and S. hydruntina Caroli 1937 from Puglia (southern Italy).

Class Remipedia
Order Nectiopoda
Family Godzilliidae
Godzillius robustus Schram, Yager & Emerson, 1986

Material examined. Turks and Caicos Islands, North Caicos Island, Cottage Pond, 9 January 2019, at 24-51 m depths, 3 specimens, USNM 1524345, USNM 1524347, USNM 1524349.

Distribution. Known only from the type locality, Cottage Pond, North Caicos Island (Schram et al. 1986).


Godzillius is a remipede genus with two large and robust species distributed in the Caribbean. The other species of the genus is G. fuchsi Gonzalez, Singpiel & Schlagner, 2013, described from Dan’s Cave and Ralph’s Sink on Abaco Island, Bahamas, where it co-

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occurs with at least five additional remipede species (Dan’s Cave) (Gonzalez et al., 2013). Currently this is the only remipede genus known from both the Turks and Caicos and the Bahamas.

Family Cryptocorynetidae

Kaloketos pilosus Koenemann, Iliffe & Yager, 2004

Material examined. Turks and Caicos Islands, North Caicos Island, Cottage Pond, 7 & 9 January 2019, at 24-51 m depths, 2 specimens, USNM 1524329, USNM 1524342.

Distribution. Known only from the type locality, Cottage Pond, North Caicos Island (Koenemann et al., 2004).

Remarks. Stygobitic. Observed swimming in the water column, together with the remipedes G. robustus and K. pilosus in deeper cave waters with total darkness and fully haline salinities. Lasionectes entrichoma is by far the most abundant remipede species collected on this current expedition, particularly in Cottage Pond. This species was absent from Airport Cave, from where it has previously been reported, likely due to water pollution.

Family Micropacteridae

Micropacter yagerae Koenemann, Iliffe & van der Ham, 2007

Material examined. Turks and Caicos Islands, Providenciales, Old Blue Hill Cave, 5 January 2019, at 22 m depth, 2 specimens, USNM 1524317, USNM 1524322.

Distribution. Known only from its type locality, Airport Cave, and Old Blue Hill, Providenciales.

Remarks. Stygobitic. Observed swimming in the water column with the remipede L. entrichoma. Micropacter yagerae was not collected at its type locality (Airport Cave) during this expedition, which we found degraded and completely depleted of life due to water pollution.

The generic name Micropacter means ‘tiny hunter’ and refers to the small size of the individuals, superficially resembling juveniles of the family Speleonecetidae.

Family c.f. Morlockiidae

Morlockia sp. nov.

Material examined. Turks and Caicos Islands, Middle Caicos, Conch Bar Cave, 5 & 7 January 2019, at < 0.5 m depth, 3 specimens, USNM 1524334, USNM 1524344.

Distribution. Known only from the type locality, Conch Bar Cave, Middle Caicos Island.

Remarks. Stygobitic. Our specimens are unusual for remipedes in that they were collected from brackish (21 ppt) surface waters of small, shallow pools, rather than higher salinity water in the deep interior of underwater caves. Only Speleonectes epilimniius Yager & Carpenter, 1999 has been found in similar conditions, described from a brackish pool (24-25 ppt) at the back of Major’s Cave (San Salvador, Bahamas) (Carpenter, 1999). Our specimens co-occurred with S. clarkei.

These specimens are under study, but preliminary attempts to identify the species based on both
morphism and molecular data limited to one marker (16S rRNA) have been so far inconclusive.

As in prior expeditions, we too recovered potentially new species (two annelids and one remipede), as well as new records for the Turks and Caicos. Our report of *Mesonerilla* and *Speleonerilla* (both belonging to family Nerillidae, Annelida) is of particular interest because the only annelid previously known from the Turks and Caicos caves was the polynoid scale worm, *Pelagomacellicephala*. The presence of *Mesonerilla* in Conch Bar Cave is not surprising; it is a cave-adapted genus, currently known from disjunct localities, including Yucatán Peninsula (México), the Bahamas, Bermuda, Cuba and Lanzarote (Canary Islands), secondarily adapted to feed on suspended organic matter in the water column of anchialine caves (Worsaae et al., 2019b). Currently this is the southernmost record for *Speleonerilla*. Their widespread occurrence in the Caribbean and Atlantic suggests that increased sampling efforts would likely result in the discovery of additional species from anchialine habitats elsewhere in the region.

Our most unexpected discovery of this expedition was the putative new remipede species from Conch Bar Cave, Middle Caicos. Surprisingly, specimens were found in small pools less than 1 m deep. These collections all happened during an incoming tide, reiterating the importance and presence of a spelean corridor, permitting unhindered movements throughout the carbonate platform. Occasionally other species have also been found in shallow anchialine cave pool habitats (i.e., *Morlockia ondinae* Garcia-Valdecasas, 1984 in Lanzarote, Martínez pers. obs.). This is the first record of a remipede from Conch Bar Cave and from the island of Middle Caicos, making it the fifth remipede species for the Turks and Caicos Islands.

Similar to other Caribbean localities (i.e., Yucatán Peninsula and the Bahamas), several of the anchialine species found in this expedition are known from multiple Caicos Islands (i.e., Providenciales, Middle, North) or have broader distributions across the Caribbean and Atlantic (see Supplemental Table). Diving collections from the Caicos Caves III expedition has extended the known range of *P. iliffei* within the Caicos Bank, originally described from shallow cave pools in Conch Bar Cave, Middle Caicos, to now also inhabit deep cave waters in Cottage Pond, North Caicos Island. While specimens from both localities are not currently available for genetic comparisons, morphologically, these annelids appear identical. It remains unknown what degree of connectivity exists between the Turks and Caicos Islands; however, Gonzalez et al., (2017) showed evidence of an active spelean corridor on the island of Eleuthera, Bahamas, whereby genetically identical species of *P. iliffei* were found from caves in excess of 100 km apart.

**DISCUSSION**

Since the first scientific expedition to the Turks and Caicos Islands in 1975, a total of 32 species of animals have been recorded from aquatic subterranean environments. The Caicos Caves III expedition recorded three additional species, including two new annelids and a new remipede, bringing the total number of known anchialine fauna in the Turks and Caicos to 35, 22 of which are known stygobites. Four of these records lack any formal identification or detailed description, but we have included them as they are historical records that were listed during description of accompanying fauna (Stock & Vermeulen, 1982; Koenemann et al., 2004), providing important information regarding the diversity that these systems sustain (see Supplemental Table). These records include one mollusk (Coeloniidae) and three copepod crustaceans (Oithonidae, Pseudodiaptomidae, Ridgewayiidae; Supplemental Table). Some of this unknown diversity had been collected from hand-dug wells breaching the groundwater, which are known to additionally host epigean freshwater dispersalist species (see for example Pesce 1985; Bowman 1986). Recent expeditions, including Caicos Caves III, omitted sampling of this type due to sediment infilling of the wells and their limited access to subsurface haline waters, possibly missing numerous representatives (mainly crustaceans) throughout the Turks and Caicos.

Fig. 5. Remipedes of the Turks and Caicos Islands. A) Godzillius robustus; B) Kaloketos pilosus; C) Lasionectes entrichoma; D) Micropacter yagerae; E) Morlockia sp. nov. All photos by Jørgen Olesen. Images not to scale.
The idea of a spelean corridor is not new – originally and somewhat flippantly suggested by Chace & Hobbs (1969), the idea was largely overlooked or dismissed until recently. Nevertheless, continued faunal surveys incorporating molecular methods (Alvarez et al., 2015) have shown this concept to be a viable means towards the broad, regional distributions seen in anchialine fauna (Santos, 2006; Hunter et al., 2008; Botello & Álvarez, 2010). In the Turks and Caicos, several crustaceans are also likely utilizing such means for distribution, including Agostocaris williamsi, Barbouria cubensis, Lasionectes entrichoma, Stygiomysis clarkei, Spelaeoniscippe provo and Typhlatya garciai, all of which are found in multiple island and cave localities on the Caicos Bank. Similar to P. iliffei, some of these crustaceans are also known from locations outside the Turks and Caicos Islands, including the Bahamas, Yucatán Peninsula, Cuba, Jamaica and the Lesser Antilles, depending on the species. While phylogeographic analyses are lacking for many of these species, it is likely that they comprise different evolutionary histories related to the presence of certain functional traits (i.e., larvae, body size, salinity tolerance) and the age of each lineage.

Considering the new records presented herein, it is evident that substantial undocumented diversity is still to be found in the anchialine realm. New species are constantly described even in relatively well-investigated and easily accessible anchialine systems, including for example those in Yucatán Peninsula and Lanzarote, where six (Rubio et al., 2015; Suárez-Morales et al., 2017b, 2017a; Angyal et al., 2018; Grego et al., 2019; Sánchez & Martínez, 2019) and five new species (Martínez et al., 2016b; Gobert et al., 2019; Worsaae et al., 2019a, 2019b), respectively, have been described within the last five years. These numbers highlight the importance of continued exploration, particularly considering that some anchialine species may use different habitats rather than caves, appearing only occasionally in caves (Martínez et al., 2013). While none of the anchialine species in the Turks and Caicos are currently protected, their small populations, relatively low densities and endemism are sufficient reasons to encourage this situation to change. In addition, a better understanding of anchialine communities would allow us to better predict how these presumably isolated but delicate ecosystems will respond to ongoing environmental change and human impacts (Mammola et al., 2019).

**Threats to Caicos Caves and its Biodiversity**

Caves are rare and fragile environments that once damaged or destroyed, are lost forever. Low densities of endemic, blind and depigmented organisms, often known from only a single location, are constantly at risk or under threats of extinction (Mammola et al., 2019). Cave waters are particularly susceptible to anthropogenic disturbances as they already exhibit extreme physiochemistries, being characterized as lightless, oxygen and food deprived habitats (Gonzalez et al., 2011). Cave animals living in these environments are often forced to switch for short periods of time to less efficient anaerobic metabolism in order to survive periods of low oxygen stress (Bishop et al., 2004). These stressors vary in form, but generally, subterranean ground waters are highly susceptible to pollution and contamination through inadequate wastewater disposal, leeching from solid waste landfills or unsanitary use of caves and groundwater (Iliffe, 1979; Martínez et al., 2020).

In 2013, Iliffe became aware that an extension of the Providenciales Airport Parking area was planned near Airport Cave. Previous diving explorations and biological collections from Airport Cave on Providenciales in 1982, 1983 and 2003 resulted in the discovery of five new species of cave-adapted crustaceans making it one of the most biologically significant caves in the country. Letters to governmental departments explaining the importance of this cave and its unique fauna were sent, urging steps be taken to protect and preserve this cave site since the property is owned by the Airport Authority. Completion of the parking area occurred as planned, yet with no obvious or immediate disturbances to the area around the cave entrance. However, upon our most recent 2019 trip to Airport Cave, the entrance pool was found to be devoid of life, likely due to exhaustion of oxygen from the water. Earlier reports several years prior to 2019 from colleagues had similarly reported foul and polluted water conditions for Airport Cave. The exact cause of the oxygen depletion is unclear, but several possibilities exist. Guano from the colony of bats nesting in the cave ceiling may have increased into the pool, resulting in an increase in bacterial utilization. Alternatively, hydrological parameters may have changed as buildup of fine, silty sediments throughout the cave caused a further reduction to subterranean groundwater circulation. However, these two issues are historical processes to caves of the Turks and Caicos Islands, and being that only Airport Cave is afflicted, it is highly suggestive of anthropogenic disturbances. Similar single cave ‘die offs’ have been known elsewhere throughout the Lucayan Archipelago (pers. obs.) and are often traceable back to changes in ground use or water pollution. Perhaps sewage from the adjacent airport complex is further polluting the groundwater, or construction of the airport’s parking lot may have blocked off underground flow channels. Until additional studies are carried out, there is no way to know for sure. Additional concerns and problems and were also observed in 2019 at Old Blue Hill Cave, the only other site on Providenciales where remipedes have been found. While this cave is located in a relatively remote part of the island, a group of people have set up a makeshift encampment, and are using the cave pool for bathing and laundering of clothes. What long-term impact this may have on water quality and the cave fauna is unclear.

In any event, cave species tend to be present in low numbers and constrained to a narrow depth range in small parts of the caves. Due to their highly restricted distribution, low population sizes and environmentally threatened habitats, most Caicos cave animals qualify for classification as endangered. As a result, subterranean environments of the Turks and Caicos Islands are valuable and in need of effective legal
protection, especially as island developments and anthropogenic encroachment continues.

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Authorship statement: All authors contributed to the collection and preservation of animals used in this study. BCG, TMI, KW and KJO designed this study. BCG, AM, JO, SBT, LB, MAL, JD, TMI and KW photographed and identified all collected material. On-site logistics, transportation, diving support and permitting were made possible through PH, MP, NM and JW. Funding was provided by KJO and BCG. BCG and AM wrote the manuscript with KJO and KW. All authors edited and approved the final draft.

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In the late fall of 1986, a team of cavers from the Gruppo Speleologico Marchigiano of Ancona reported to the regional Archeological Superintendence their discovery in a room of the Grotta del Fiume Cave (Marche region of central Italy) of archeological finds such as the charcoal remains of a bonfire, a number of ibex bones, and four chert artifacts. However, since the discovery and archival of all this material, no further research or focused studies were carried out about this important archeological site. Therefore, we engaged in a thorough re-examination of this site by mapping this part of the Grotta del Fiume Cave with modern surveying approaches, including terrestrial laser scanner and ground penetrating radar. Moreover, we have radiocarbon dated a few ibex bones and also charcoal samples from the main fireplace and other smaller charcoal deposits we found scattered around the room's floor. The chert artifacts were examined in detail and their typology classified accordingly. U-Th dating of calcite from speleothems helped us reconstruct this hypogeal environment at the time it was frequented by man. We also studied a puzzling deposit of speleothem crust slabs piled up in a corner of the cave, which may represent a funeral earthen mound. All in all, the absence of artifacts other than the four chert blades, the few ibex bones, the one main fireplace and three other minor charcoal deposits in the room, and the probable earthen mound located tens of meters underground from a now debris-sealed entrance, lead us to hypothesize that this cave room does not represent a residential dwelling but rather a worship place sporadically utilized by few representatives of a local Epigravettian hunter community.

An Epigravettian hypogeal site in the Grotta del Fiume Cave at Frasassi (northeastern Apennines, Italy): Environmental and geochronologic assessments

Alessandro Montanari, Artur Adamek, Angelo Curatolo, Marco P. Ferretti, Maurizio Mainiero, Sandro Mariani, David McGee, Gaia Pignocchi, and Stefano Recanatini

Abstract: In the late fall of 1986, a team of cavers from the Gruppo Speleologico Marchigiano of Ancona reported to the regional Archeological Superintendence their discovery in a room of the Grotta del Fiume Cave (Marche region of central Italy) of archeological finds such as the charcoal remains of a bonfire, a number of ibex bones, and four chert artifacts. However, since the discovery and archival of all this material, no further research or focused studies were carried out about this important archeological site. Therefore, we engaged in a thorough re-examination of this site by mapping this part of the Grotta del Fiume Cave with modern surveying approaches, including terrestrial laser scanner and ground penetrating radar. Moreover, we have radiocarbon dated a few ibex bones and also charcoal samples from the main fireplace and other smaller charcoal deposits we found scattered around the room's floor. The chert artifacts were examined in detail and their typology classified accordingly. U-Th dating of calcite from speleothems helped us reconstruct this hypogeal environment at the time it was frequented by man. We also studied a puzzling deposit of speleothem crust slabs piled up in a corner of the cave, which may represent a funeral earthen mound. All in all, the absence of artifacts other than the four chert blades, the few ibex bones, the one main fireplace and three other minor charcoal deposits in the room, and the probable earthen mound located tens of meters underground from a now debris-sealed entrance, lead us to hypothesize that this cave room does not represent a residential dwelling but rather a worship place sporadically utilized by few representatives of a local Epigravettian hunter community.

Keywords: Frasassi caves, archeology, Paleolithic fireplace, Late Pleistocene Ibex, Epigravettian lithic industry

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INTRODUCTION

On November 15, 1986, while exploring the Grotta del Fiume Cave in the Frasassi Gorge (Marche region, northeastern Apennines of Italy), a team of cavers from the speleologic group Gruppo Speleologico Marchigiano (GSM) of Ancona, discovered a new room christened by them “Galleria dello Stambecco” (Gallery of the Steinbock), and here referred to as “Sala del Fuoco” (Room of Fire). In this remote and difficult-to-reach room, the cavers found a number of animal bones, the charcoal remains of a fireplace, and, most striking, the skull of a mountain goat or steinbock (Capra ibex; ibex from hereafter) set on the tip of a short stalagmite (Fig. 1). These finds were dutifully reported to the Archeological Superintendence of Marche on December 21 of the same year by Giuseppe Antonini, one of the discoverers and at the time
President of the GSM of Ancona (Antonini, 1986). On October 30, 1987, Forestalp, a cooperative firm operating outdoor activities such as on-rope aerial jobs, of which Antonini was an executive member, terminated the survey of Galleria dello Stambecco (cf. Sala del Fuoco), and delivered to the Archeological Superintendence of Marche a report, which described in detail the techniques and methods used for surveying the site, and for drafting a map with the location of the various archeological finds. Forestalp’s report emphasized that the cave was located no more than 10 m below the surface but the access to the outside at the end of a narrow tunnel was obstructed by debris. The report also reiterated the original hypothesis by Antonini (1986) that the cave did not represent a residential dwelling but rather a prehistoric place of worship. On November 15 of the same year, Forestalp (1987) asked permission from the Archeological Superintendence to carry out the recovery of archeological finds in Sala del Fuoco, which included: 1) “chert artifacts”; 2) “steinbock skulls and animal bones belonging to various individuals”; and 3) “fossilized charcoal pieces”. Permission was granted by the chief superintendent on November 27 of the same year. Eventually, the recovered archeological material along with the Forestalp report was archived and stored away by the in-charge official of the Archeological Superintendence, on January 29, 1990. Unfortunately, of all the material provided by Forestalp, only a box containing four chert artifacts is to be found today in the archive of the Archeological Superintendence. Regrettably, the map and photographs, the fireplace charcoal sample, the abundant bones, and the ibex skull(s) were not to be found anywhere in the archive, and no indication of where else they may have been stored was given to us by the archive’s attendants.

Despite its importance, the discovery of Sala del Fuoco with its little archeological treasure was never publicized, and, as far as we know, no further scientific investigations were made nor published about this site. Twenty years after being discovered, we revisited this room while studying alluvial sediments (clay, sand, and gravel) often found in near-entrance sites in the Frasassi cave complex (e.g., Mariani, 2003; Pignocchi & Montanari, 2016; Montanari et al., 2019). At about the same time, during a study involving radiocarbon dating of Holocene subfossil eels grounded at different heights above present water table in a remote inner region of the Grotta del Fiume Cave, we had the chance to have a sample of the fireplace charcoal radiocarbon dated with AMS by Klaas van der Borg (Utrecht University), a coauthor in Mariani et al. (2007). The charcoal yielded a radiocarbon age of 13,550 ± 90 yr later calibrated at calendar age 16,646-16,046 yr cal. BP (Fig. 2), which was recently mentioned in a few abstracts (Pignocchi, 2015; Montanari et al., 2017) and in a conference proceedings volume (Pignocchi et al. 2018). Our preliminary date of ~16.3 ka for the fireplace and the associated chert artifacts in Sala del Fuoco represented one of the few radioisotopically dated evidence for an Epigravettian culture in this region of the northern Apennines (Broglio et al., 2005; Silvestrini et al., 2005, 2008), and one of the few dated archeological sites in Italy belonging to the Late Pleniglacial Stage (e.g., Ravazzi et al., 2007; Cremona, 2008; Mussi et al., 2008, and references therein). We therefore engaged in a thorough environmental study of this site including cartographic and terrestrial laser scanner (TLS) mapping, speleo-geomorphologic, and sedimentologic study of the Sala del Fuoco including a ground penetrating radar survey (GPR), speleothem...
U-Th dating, and $^{14}$C AMS dating of ibex bones and charcoal samples from the main fireplace and from three other small charcoal deposits we found in different places in the cave room. We also studied, and wondered about, a puzzling deposit of speleothemic crust slabs piled up on one side of the room not far from the fireplace, which may represent an earthen mound.

**METHODS AND TECHNIQUES**

The topographic survey of the Sala del Fuoco room in the Grotta del Fiume cave was accomplished with a combination of closed and open polygonals, a method traditionally used in cave mapping. It consists in making a polygonal line along the perimetral walls of a cave room or through a narrow tunnel, taking measurements with a compass for reading horizontal angles (direction), clinometer for vertical ones (inclination), and measuring tape for determining distances (length). In our case we used a laser instrument called DISTOX2. It is a Leica Disto X310 modified with an upgrade kit, which includes a 3-axis compass, a clinometer, and a Bluetooth connection. For the 3D survey of Sala del Fuoco we used a Terrestrial Laser Scanner FARO FOCUS 3D for acquisition data, and the FARO SCENE software for elaborating the acquired point clouds.

Ground-penetrating radar (GPR) is a non-destructive geophysical method that uses radar pulses to image the subsurface using electromagnetic radiation in the microwave band (UHF/VHF frequencies) of the radio spectrum, and detects the reflected signals from subsurface structures. For the survey of Sala del Fuoco we used a ZOND 12E GPR RADAR with an 1.5 Ghz antenna and signal acquisition at 100 ns.

About 200 high-resolution photographs were taken in Sala del Fuoco using a CANON EOS 5D MARK II digital camera to document speleo-geomorphologic and environmental features, and close-up pictures of sampled sites. After locating the sampled sites on the map, and having photographed them, sampling proceeded using tweezers for picking up charcoal fragments, finger picking to collect bones, and a trowel to collect loose sediment.

Radiocarbon AMS dating of charcoal, bone, and speleothemic calcite samples was performed at the Beta Analytic Laboratory in Miami, (USA), using sample preparation and pretreatment routinely applied for these materials by this laboratory.

Samples of speleothemic calcite were collected using hammer and chisel, and later cut in centimetric subsamples with a wet diamond rock saw for U-Th dating in the McGee Laboratory at Massachusetts Institute of Technology. We used the U-Th chemistry procedure of Edwards et al. (1987) to prepare the samples. This procedure included addition of a calibrated $^{230}$Th/$^{234}$U-$^{238}$U tracer solution, dissolution, iron co-precipitation, and anion exchange chromatography in order to isolate the small amounts of U and Th from the calcium carbonate. The U and Th isotopes were measured using the Nu Plasma II multicollector-inductively coupled plasma–mass spectrometer (MC ICP-MS) with enhanced sensitivity (ES) interface in the McGee Laboratory. U-Th ages were calculated using the decay constants determined by Jaffey et al. (1971) for $^{238}$U and Cheng et al. (2013) for $^{235}$U and $^{239}$Th. Corrections for initial $^{230}$Th assumed an initial $^{230}$Th/$^{232}$Th atomic ratio of $4.4 \pm 2.2 \times 10^{-6}$ (Taylor & McLennan, 1985).

**SPELEO-GEOLOGIC SETTING**

The lower Jurassic carbonate platform limestone of the Calcare Massiccio Formation making up the core of the Valmontagnana-Frasassi blind thrust anticline in the Marche Apennines of central Italy, hosts the Frasassi karst complex (Fig. 3 A-B), which counts a dozen or so natural caves and caverns as registered in the regional cadaster. The largest interconnected cave system of the Frasassi complex is represented by the Grotta Grande del Vento (now a world-famous tourist show cave) and the Grotta del Fiume caves, both located in the south side of the Frasassi Gorge with a total development of about 25 km (Fig. 3A). The architecture of this cave system is characterized by seven subhorizontal levels (Bocchini & Coltorti, 1978), which are connected by nearly vertical shafts. Of these hypogeeal levels, the 3rd and the 5th are the best developed, while the 1st level represents the local phreatic base level at about 200-205 m above sea level (asl). However, recent speleosubc exploration of some hypogeeal lakes revealed an astonishing maze of large caverns extending subhorizontally within the saturated phreatic zone down to depths of ~30 m below base level (Jenn Macalady, pers. comm., summer 2016). It is in this subaqueous environment where dissolution of the limestone is presently occurring due to a localized sulfide hypogenic karst process (e.g., Galdenzi et al., 1999, 2008; Forti et al., 2002; Macalady et al., 2006, 2008; Mariani et al., 2007). Hypogenesis occurs at Frasassi because sulfide waters rich in dissolved H$_2$S upwell from the Triassic evaporitic anhydrites (CaSO$_4$) of the Burano Fm., into and through the overlying Calcare Massiccio limestone via a deeply-rooted fault and associated network of fractures and joints (Fig. 3B).

The multistory architecture of the cave system is the result of alternating erosion (thus incision) of the Sentino River flowing from west to the east at the bottom of the deeply incised Frasassi Gorge during interglacial warm and wet periods, and aggradation (valley fill) in cold and dry glacial periods, all this occurring during regional tectonic uplift (e.g., Cattuto, 1976; Galdenzi, 1990; Wegmann & Pazzaglia, 2008: Montanari et al., 2019; and references therein). The dynamics of tectonic uplifting coupled with river incision implies that the upper levels of the cave system are the oldest (e.g., Taddeucci et al., 1992, 1994; Montanari et al., 2019) but it also means that, Frasassi being a hypogenic karst, the river will eventually cut the cave spaces that are currently forming several meters below its thalweg, which, in fact, represents the local base level. It is so that the craggy sides of the Frasassi Gorge are studied...
Fig. 3. Simplified geologic map (A) and cross section (B) of the Frasassi blind thrust-anticline (modified after Mariani et al., 2007), showing the location of the Frasassi hypogenic-karst cave complex incised by the Sentino River. The white-framed area of Grotta del Fiume in (A), with indicated the location of Sala del Fuoco (SDF), refers to Fig. 4B.

with cave apertures, caverns, and recesses (Fig. 3A) especially on the eastern end of the gorge. This is where the Frasassi cave complex develops on a NW-SE general trend along a strongly fractured Calcari Massiccio limestone on the footwall of a reactivated Jurassic normal master fault (Fig. 3B), which, on the Valmontagnana side of the gorge, runs along a steep gully locally known as Fosso Riccio (Fig. 4A).

For a precise location of Sala del Fuoco and Galleria dello Stambecco with respect to the topography of the Fosso Riccio fault zone, we have joined our topographic survey to the original map of Grotta del Fiume of Bocchini & Coltorti (1978), as shown in Fig. 4B. Sala del Fuoco turns out to be located at an elevation of 265 m asl (i.e., about 65 m above present base level), thus representing the well-developed 5th level of the cave system, which was incised by the Sentino River probably in the late Middle Pleistocene around 200 ka (Cattuto, 1976; Taddeucci et al., 1992, 1994; Montanari et al., 2019, and references therein). In summary, the abundance of rupestrian caves found on the slopes and cliffs of the Frasassi Gorge is a geomorphic feature peculiar to this area in the context of the northern Apennine karst, and uniquely related to the coincidence of the localized sulfidic hypogenic karst process and the Pleistocene-Holocene regional geotectonic activity (uplifting and incision).
ENVIRONMENTAL AND GEOCHRONOLOGIC ASSESSMENTS

Hypogean environment

Through the years, in the Frasassi area archeologists have found evidence of human dwelling in a few caves, and radiocarbon dating indicated a frequentation period between 13,000 and 11,500 yr cal. BP (final Epigravettian; Pignocchi, 2015, 2018, and references therein). A number of archeological digs in the large Grotta della Beata Vergine Cave on the northwestern side of the Frasassi Gorge (see GBV in Fig. 3A) from the second half of the 19th century through the first half of the 20th century (Pignocchi & Montanari, 2016, and references therein), yielded evidence of its utilization for funeral purposes from Early Bronze Age to medieval times, but no evidence ever emerged for pre-Neolithic frequentation other than the fortuitous find, by a casual visitor, of a Venus figurine of dubious Gravettian age (Coltorti et al., 2012; Pignocchi & Montanari, 2016). Therefore, the fireplace in Sala del Fuoco in the Grotta del Fiume Cave preliminarily dated at about 16,300 yr cal. BP (Fig. 2), and the associated chert artifacts and ibex bones reported by Antonini (1986), represent the oldest evidence of Homo sapiens in a cave in this region of the northern Apennines.

In the Frasassi area, prehistoric finds were unearthed in relatively small and shallow cave shelters where daylight could diffuse directly in the dwelling (Broglio & Lollini, 1982; Bartolomei & Cattani, 2005). On the other hand, the vast cavern of Sala del Fuoco is located at such a distance from a probably narrow entrance that daylight could not reach. With the help of CB portable radios and ARVA avalanche transceiver, we have located within 5 m in the Fosso Riccio gully at about 265 m asl (Fig. 4A, B) the northeastern end of a narrow tunnel, which likely represented the access to Sala del Fuoco now obstructed by scree (Site 9B in Fig. 5A). Inside the cave, the vicinity of the surface was indicated by plant roots sticking out of the scree, and also by the presence of insects and a numerous population of salamander Speleomantes italicus. In the Moonmilk Room (Site 10 in Fig. 5A and B), which contains the largest...
deposit of fresh moonmilk (i.e., moist and still forming) ever found in any of the Frasassi caves, we have counted up to 64 clustered individuals of this troglobite amphibian at once. The scree itself was made up of a fine uncemented breccia (Fig. 6B) composed of angular clasts of multicolored limestones and cherts derived from various lower Cretaceous to Eocene pelagic carbonate formations, which are at present exposed at the top of Monte Valmontagnana (see Figs. 3A and 4A for location). Outside, the steep and densely forested Fosso Riccio gully did not offer any visible cave entrance anywhere around the precise point where we were getting the ARVA and CB radio signals loud and clear. Nevertheless, the soil of the gully turned out to be made up of a pedogenized fine polymictic breccia compositionally identical to the breccia that fills up the tunnel of Sala del Fuoco in Site 9B.

From the Moonmilk Room, going SW, one enters into the wide space of Sala del Fuoco, a flat bottom horizontal room some 50 m long, 20-30 m wide, and up to 10-15 m high, beautifully decorated with stalagmites of various colors (from white to oranges, beiges, and grays), and sizes (Figs. 5B and 6A). The ceiling and the eastern wall are mostly white in color due to a widespread coat of dry moonmilk. Stalactites are few, and are more numerous in the northern part of the room. Some of them have a dull appearance with a beige tinge, some others are whitish due to a coat of dry moonmilk. Overall, Sala del Fuoco appears quite dry at present with almost no active dripping. The floor is locally covered by limestone angular clasts of various sizes possibly resulting from stripping of the vault due to cryogenic processes and/or seismic activity, and often encrusted by speleothemic calcite. In the case shown in Fig. 6C (Site 7 in Fig. 5A), the millimeters-thick speleothemic crust covers an intact ibex radius bone about 18 cm long, which yielded a collagen $^{13}$C AMS age of 19,002–18,732 yr cal. BP (Table 1). The calcite crust (whole thickness, see insert in Fig. 6C) was dated to 10,130 ± 480 ka by U-Th (Table 2). $^{13}$C AMS dating of the same speleothem sample yielded an age of 12,741–12,667 yr cal. BP (Table 1), ~2,500 years older than the U-Th age, likely reflecting inputs of dead carbon from the bedrock (e.g., Valladas et al., 2017). It is worth noting that the floor speleothemic crust is dusted with carbonate particles resembling snowflakes (Fig. 6C), which probably fell from the moonmilk-encrusted walls and vaults of the cave (Fig. 6C).
Fig. 6. A) Panoramic view of Sala del Fuoco indicating sites and features described in the text. Circled numbers refer to sites as in Fig. 5; fstg stands for fallen stalagmite, whereas lstg stands for leaning stalagmite; B) Close up view of the fine polymictic breccia obstructing the exit tunnel in Site 9B (see Fig. 5 A for location); C) Limestone breccia and ibex radius bone on the cave floor encrusted by speleothem calcite (Site 7). The insert shows a section of the bone with the superficially enveloping speleothem calcite crust; D) Cross section view of the cave floor made up of an alluvial deposit of moderately-to-well rounded sand grains and pebbles encrusted by speleothem calcite.

Table 1. Radiocarbon ages for charcoal, bone, and speleothem samples from Sala del Fuoco in Grotta del Fiume (Frasassi, Italy) from AMS $^{14}$C analysis.

<table>
<thead>
<tr>
<th>Lab code</th>
<th>Sample name</th>
<th>Material</th>
<th>Measured age (yr BP)*</th>
<th>$\delta^{13}$C ‰</th>
<th>Conventional age (yr BP)*</th>
<th>calendar age (yr cal BP)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>545651</td>
<td>SDF-1-COAL</td>
<td>charcoal</td>
<td>13,440±40</td>
<td>-23.5</td>
<td>13,460±30</td>
<td>16,372-16,015</td>
</tr>
<tr>
<td>545652</td>
<td>SDF-1A-COAL</td>
<td>charcoal</td>
<td>13,560±50</td>
<td>-28.8</td>
<td>13,500±50</td>
<td>16,479-16,058</td>
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<tr>
<td>545653</td>
<td>SDF-1B-COAL</td>
<td>charcoal</td>
<td>13,450±40</td>
<td>-26.9</td>
<td>13,420±40</td>
<td>16,310-15,970</td>
</tr>
<tr>
<td>545654</td>
<td>SDF-1C-COAL</td>
<td>charcoal</td>
<td>13,830±40</td>
<td>-23.3</td>
<td>13,740±40</td>
<td>16,999-16,558</td>
</tr>
<tr>
<td>465542</td>
<td>SDF-MANDIBULA</td>
<td>mandible bone</td>
<td>13,650±40</td>
<td>-19.5</td>
<td>13,740±40</td>
<td>16,835-16,362</td>
</tr>
<tr>
<td>461958</td>
<td>SDF-MAXBONE</td>
<td>radius bone</td>
<td>15,530±60</td>
<td>-19.3</td>
<td>15,620±60</td>
<td>19,002-18,732</td>
</tr>
<tr>
<td>461960</td>
<td>SDF-MAXCRUST</td>
<td>speleothem calcite</td>
<td>10,440±30</td>
<td>-4.6</td>
<td>10,770±30</td>
<td>12,741-12,667</td>
</tr>
</tbody>
</table>

Accelerator mass spectrometry (AMS) radiocarbon dating was performed at Beta Analytic Inc. (Florida) following methods and techniques in [http://www.radiocarbon.com/accelerator-mass-spectrometry.htm](http://www.radiocarbon.com/accelerator-mass-spectrometry.htm).

*Dates are reported as radiocarbon years before present, where “present” = AD 1950.
Table 2. Results from U-Th analysis of speleothemic calcite samples from Sala del Fuoco of Grotta del Fiume (Frasassi, Italy).

<table>
<thead>
<tr>
<th>MIT McGee Lab</th>
<th>Sample description</th>
<th>238U (ng/g)</th>
<th>± (2σ)</th>
<th>232Th (ng/g)</th>
<th>± (2σ)</th>
<th>δ234U (ppm atomic)</th>
<th>± (2σ)</th>
<th>230Th/238U activity</th>
<th>± (2σ)</th>
<th>Age (yr)</th>
<th>± (2σ)</th>
<th>δ234U initial (pg/g)</th>
<th>± (2σ)</th>
<th>Age (yr BP)</th>
<th>± (2σ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>SDF-BST-A</td>
<td>stalagmite fatg83-A</td>
<td>39</td>
<td>1</td>
<td>129</td>
<td>3</td>
<td>0.1227</td>
<td>0.0014</td>
<td>590</td>
<td>7</td>
<td>11030</td>
<td>130</td>
<td>279</td>
<td>2</td>
<td>10880</td>
<td>130</td>
</tr>
<tr>
<td>SDF-BST-B</td>
<td>stalagmite fatg83-B</td>
<td>33</td>
<td>1</td>
<td>445</td>
<td>9</td>
<td>0.1280</td>
<td>0.0020</td>
<td>152</td>
<td>2</td>
<td>11110</td>
<td>190</td>
<td>327</td>
<td>2</td>
<td>10740</td>
<td>240</td>
</tr>
<tr>
<td>SDF-BST-C</td>
<td>stalagmite fatg83-C</td>
<td>30</td>
<td>1</td>
<td>288</td>
<td>6</td>
<td>0.1149</td>
<td>0.0015</td>
<td>189</td>
<td>3</td>
<td>10190</td>
<td>140</td>
<td>292</td>
<td>2</td>
<td>9900</td>
<td>180</td>
</tr>
<tr>
<td>SDF-BST-D</td>
<td>stalagmite fatg83-D</td>
<td>30</td>
<td>1</td>
<td>2930</td>
<td>60</td>
<td>0.0750</td>
<td>0.0017</td>
<td>12</td>
<td>0</td>
<td>6330</td>
<td>150</td>
<td>330</td>
<td>2</td>
<td>4000</td>
<td>1200</td>
</tr>
<tr>
<td>SDF-MAXCRUST</td>
<td>crust over radius bone</td>
<td>42</td>
<td>1</td>
<td>1890</td>
<td>40</td>
<td>0.1413</td>
<td>0.0013</td>
<td>50</td>
<td>1</td>
<td>11130</td>
<td>110</td>
<td>463</td>
<td>2</td>
<td>10130</td>
<td>480</td>
</tr>
<tr>
<td>SDF-FLOORSLAB</td>
<td>slab from the Tumulus</td>
<td>24</td>
<td>1</td>
<td>4120</td>
<td>80</td>
<td>0.8110</td>
<td>0.0050</td>
<td>74</td>
<td>1</td>
<td>103200</td>
<td>1100</td>
<td>379</td>
<td>4</td>
<td>99300</td>
<td>2300</td>
</tr>
</tbody>
</table>

*Reported errors for 238U and 232Th concentrations are estimated to be ±1% due to uncertainties in spike concentration; analytical uncertainties are smaller;
\[ \Delta [238U] = (\text{[238U]} - \text{[238U]}_{\text{activity}}) \times 1000; \]
\[ [238U]_{\text{activity}} = 1 - e^{-230Th/238U} \times \left( \frac{\delta 234U_{\text{measured}}}{1000} \right)^{\frac{\lambda_{230}}{\lambda_{230} - \lambda_{234}}}; \] where T is the age. “Uncorrected” indicates that no correction has been made for initial 230Th;
\[ \delta 234U_{\text{initial corrected}} = \delta 234U_{\text{measured}} \times X e^{\chi 234}, \] where T is corrected age;
*Ages are corrected for detrital 230Th assuming an initial 230Th/232Th of (4.4 ± 2.2) × 10^-6;
*BP stands for “Before Present” where the “Present” is defined as the January 1, 1950 C.E;
Decay constants for 230Th and 234U are from Cheng et al. (2013); decay constant for 238U is 1.55125 x 10^-10 yr^-1 (Jaffey et al., 1971).
In other places throughout the cavern, the floor calcite crust covers an alluvial slack water deposit made up of moderately-to-well rounded sand grains and pebbles (Fig. 6D) of varied lithology, which represent the formations of the Jurassic to Miocene Umbria-Marche sedimentary succession, practically the same lithologic composition of the sediment making up Pleistocene fluvial terraces as well as the present-day Sentino river bed. As for the stalagmites, they range from a few cm to a few m in height. The tallest of all, with an estimated height of 5-6 m and a diameter at the base of about 1 m, is the one located at the southern end of the room next to the fireplace (Site 2 in Fig. 5A, B and visible in the far distance of panoramic view in Fig. 6A), and here christened the “Totem”. Interestingly, half a dozen or so relatively large broken stalagmites are laying down on the floor or leaning against the eastern side wall of the cave, with a common NW-SE axial trend (Figs. 5A and 6A). This suggests that these fallen stalagmites were knocked down by a violent seismic shear wave, which propagated from an epicenter that was located either to the SE or to the NW of Sala del Fuoco.

In order to envision how the environment of Sala del Fuoco may have looked like some 16,300 years ago, it would be necessary to determine the age of the speleothems, and assess which ones were already present there when this hypogeal room was visited by humans. Intuitively, small, slim, fresh looking stalagmites are younger than larger and taller ones, but this could well be a sidetracking intuition. A 5 cm stalagmite growing on a chert artifact found by Forestalp (1987) in Sala del Fuoco turned out to be 1,500 ± 400 yr old after U-Th dating by Taddeucci et al. (1992, 1994). The oldest U-Th date of 199 ± 21 ka ever obtained from a speleothem from the 5th level of the Grotta del Fiume Cave (i.e., Sala del Limone, at about 265 m asl) was from the base of a 30-cm-tall stalagmite (Taddeucci et al., 1992). With the intention of contributing to the reconstruction of the speleothemic history of this cave, we have U-Th dated a 83-cm-long broken piece of a stalagmite (sample SDF-fstg83; Fig. 7A), probably taller than 130 cm in origin before it was knocked down from its 50 cm stump. This fallen stalagmite now rests head-down with a NW-SE axial trend over an intriguing pile of broken speleothemic slabs (Fig. 7A; for location, see Site 4 in Figs. 5A and 6A). The facing-up side of the stalagmite is covered by a thin layer of finely laminated gray speleothem (Fig. 7B), which was deposited from localized dripping after it fell. Also the piled up slabs are locally encrusted by the same gray speleothemic cover (Fig. 7C). An age of about 10.8 ka for the oldest part of the 21 cm long stalagmite piece we sampled, and an age of about 9.9 ka for the youngest part of it (Table 2; Fig. 7B), suggest a mean growth rate of 0.02 mm yr⁻¹. Considering that the basal stump still in place is about 50 cm tall, this suggests that the stalagmite started to grow way before the end of the last glacial. On the other hand, the gray laminated speleothem covering the facing up side of the fallen stalagmite yielded a U-Th age of 4,000 ± 1,200 years (Table 2; Fig. 7B), which suggests that the stalagmite fell down over the slab pile not later than 4-5 thousand years ago. Of course direct dating of the base and tip of the whole stalagmite will eventually give a more accurate geochronology of its growth history, and a better-constrained date of its fall. On the other hand, a piled up speleothem slab yielded an age of 99.3 ± 2.3 ka (Table 2; Fig. 7C), suggesting that speleothemic concretion was going on already tens of thousand years before Sala del
Fuoco was visited by humans. Nevertheless, these few educated observations corroborated by radioisotopic geochronology induce us to envision a Sala del Fuoco around 16 thousand years ago much more bare of speleothems than it is today. The 5-m-tall Totem was probably one of the few conspicuous speleothems decorating this hypogeal environment, and likely the most imposing.

**Quest for fire**

The remains of a fireplace at the foot of the Totem giant stalagmite (Fig. 8A; Site 1 in Fig. 5), consist of small charcoal particles distributed on the floor in a roughly circular area about 1 m in diameter (Fig 8B). In this site, the floor is made up of uncemented limestone breccia. When one of us (S. M.) first visited Sala del Fuoco in 2002, he found the fireplace marked by limestone and speleothem boulders arranged in a circle around the burnt ground. The rocks were placed there probably by earlier cavers with the considerate intention of protecting the site from possibly passing-by inattentive spelunkers. Among the burnt limestone breccia (including a few broken pieces of stalactites), we did not notice any bone remains but only black carbon particles. The flowstone “bib”, which festoons all around the base of the Totem, is clearly blackened in the sector adjacent to the fireplace (Fig. 8B), whereas everywhere else it is whitish in color. The Totem itself exhibits a pristine white color. On the other hand, the cave wall next to the fireplace is spotted with a brownish stain up to several meters above the floor, which contrasts with the otherwise white color of the dry moonmilk that coats the vaults of the cavern. This may be the result of the smoke produced by the fireplace. A more convincing evidence for smoking of the environment around the fireplace is given by a micron-thick veneer of actual black soot, which covers a clay deposit (i.e., a clay-rich slack water sediment) found along the Galleria dello Stambecco (Fig. 8A, and Fig. 5A for location). This long tunnel puts Sala del Fuoco, and in particular the fireplace area, in direct communication with the vast spaces of the inner parts of the Grotta del Fiume cave, from here immediately down to the Sala delle Ossa room (Fig. 5A for location). This may have provided some ventilation for the smoke produced by this primitive bonfire, which would have directed the smoke towards the inner part of the cave rather than in the ambience of Sala del Fuoco. In any case, a new small sample of charcoal was collected in one of our last expeditions in 2019 with the intention of replicating, in a different lab, the original 14C AMS date of 16,646-16,046 yr cal. BP (Fig. 2). In fact, the new charcoal sample yielded an age of 16,372-16,015 yr cal. BP (Table 1), slightly more precise but indistinguishable, at a 95% confidence level, from the original date.

The fireplace described above is not the only evidence of fire lighting in Sala del Fuoco. We have identified three other charcoal deposits in this cave room, two small ones next to the eastern wall of the cave (Sites 1A and 1B in Fig. 5A), and a larger third one on the central-western side of the room next to the edge of the 15-m-deep vertical shaft that drops down to Sala delle Ossa (Site 1C in Fig. 5A).
Site 1A consists of a sprinkle of charcoal particles resting on top of a fine polymictic alluvial gravel similar to the one shown in Fig. 6D. It must be pointed out, however, that this charcoal deposit may have been spread over a larger area around the presently exposed find, which is now covered by a speleothemic crust and a group of small stalagmites (Fig. 8C). A sample of this charcoal yielded an age of 16,479-16,058 yr cal. BP, thus indistinguishable from the age of the charcoal from the main fireplace (Table 1).

Site 1B is located about 5 m from Site 1A (Fig. 5A for location). As in the previous case, charcoal particles lay on polymictic alluvial gravel well exposed over a wide surface (Fig. 8D). Yet it does not consist of a single concentrated spot but sporadic charcoal particles are found aligned on a 5-6 m long track directed toward the center of the room. This distribution suggests that these charcoal finds may not represent a fireplace but possibly the sites where small spotlights or torches were lit to illuminate the room. A sample of the charcoal collected from Site 1B yielded an age of 16,310-15,970 yr cal. BP, once again indistinguishable from the age of the charcoal from the main fireplace (Table 1).

On Site 1C, charcoal specks were found sparse over a half square meter area where the floor is made up of cemented limestone breccia dusted with moonmilk particles (Fig. 8E), a situation similar to the one seen on Site 7 shown in Figure 6E. However, close observation revealed that the limestone clasts around the exposed (visible) charcoal particles are darker than those making up the floor of the surrounding area, suggesting that they have been baked by fire. It is worth mentioning that at a short distance from this smaller fireplace, we found a blob of what looked like red terracotta. Examination under the microscope revealed that this material was indeed a blob of clay, which had been baked in an open fire. As a simple test, we produced a very similar material by baking in a house fireplace a blob of a clay sample collected from the aforementioned Galleria dello Stambecco. These observations lead to the hypothesis that this was the site of another fireplace similar to the one found at the aforementioned Galleria dello Stambecco. These observations lead to the hypothesis that this was the site of another fireplace similar to the one found at the foot of the Totem at the very end of the room but this time placed at the edge of a gloomy chasm, i.e., the northeastern 15-m-tall vertical wall of Sala delle Ossa. A sample of the charcoal collected from this site yielded a ^14C AMS age of 16,999-16,558 yr cal. BP (Table 1). This age, equivalent to ~16.8 ± 0.2 ka, is several hundred years older than a mean age of ~16.3 ± 0.2 ka obtained from the other charcoal samples.

The steinbock ossuary

In August 1971, a team of cavers from Gruppo Speleologico Città di Jesi passed through a bottleneck and consequently the bone specimens described by Coltorti & Sala (1978) cannot be traced back today. On the other hand, we can say with hindsight that there are no sinkhole openings on the ceiling of Sala delle Ossa but only a ~15 m high vertical wall, which climbs up to the overhanging western edge of Sala del Fuoco (see Fig. 5A for location), where, in fact, more ibex bones were discovered by the cavers of the GSM of Ancona 15 years later (Antonini, 1986). Apart from the isolated radius we found encrusted on the floor of Site 7 (Fig. 6E), and the ibex skull found on the tip of a stalactite, of which the sole evidence of its existence is the photograph taken by an unknown caver in 1986 (Fig. 1) as published in Parco Naturale Regionale della Rossa e di Frasassi (2000), and also the “numerous bones” originally reported by Antonini (1986) and Forestalp (1987), only a handful of mostly broken bones were found by us in Sala del Fuoco, beneath the cave wall in Site 8A (see Fig. 5A for location). They were actually distributed in two separate groups about 40 cm from each other (Fig. 9A). In addition to these, a single piece of a long bone was found on Site 8B, just a few meters from Site 8A in Figure 5A (see bone n. 17 in Fig. 9B). At first glance, it appeared that the bones arranged in that way in Site 8A were not representing the leftovers of a predator’s meal, like that of a fox, a wolf, or a big cat, which, unless they are clustered around a scavenged carcass, are usually found spread over a much larger area. The bone fragments were of different kinds (long limb bones, short bones such as vertebrae, flat bones such as ribs) ranging in length from a few to several cm. After having taken high-resolution photographs of this site, we collected all the bone fragments we could find (about 70 pieces in total; Fig. 9B and C), took close up pictures of each specimen, and eventually we dutifully delivered the whole collection to the Archeological Superintendence in Ancona on December 12, 2017.

One of the most conspicuous specimens we collected is the central portion of a left mandible of a bovid (i.e., compatible with an ibex) with a dp4 (fourth deciduous premolar) and m1 (first molar) still in place in their alveola. The presence of a milk tooth and the almost unworn first molar, indicate that the animal was not...
Fig. 9. A) Close up view of the two groups of bones as we found them in Site 8A in Fig. 5; B) Selection of diagnostic ibex bones (1 to 12) from Site 8A, and bone n. 13 from Site 8B: 1) fragment of left mandible with dp4 and m1 (lingual view); 2) fragment of lower molar, probably m2; 3) root of incisor tooth; 4) and 5) thoracic vertebrae; 6) fragment of scapula showing a possible tooth mark; 7) unfused olecranic process of ulna; 8) diaphysis of radius; 9) right metacarpal; 10) diaphysis of ulna; 11) proximal end of metatarsal; 12) right astragalus; 13) diaphysis of tibial; C) All non-diagnostic ibex bone fragments collected from Site 8A.

older than one year. Collagen from a small fragment of the lower part of the jaw yielded a $^{14}$C AMS age of 16,835–16,362 yr cal. BP, thus indistinguishable, at a 95% confidence level, from the age of the main fireplace (Table 1). All the other bones do not show traces of butchery carving, nor of masticatory snapping, except for a 3 mm circular hole in bone n. 6 (indicated in Fig. 9B), which could have been produced by a canine bite from a carnivore. Almost all long bones are fractured longitudinally (Fig. 9C), a fact that is not agreeable with the chewing action of an animal but is more compatible with the crushing of the bone with a stone, a common practice by primitive hunters trying to suck out the palatable bone marrow.

All more or less diagnostic bones found in Site 8A may represent a single, medium-size juvenile bovid, probably a one-year-old ibex. This assessment is suggested by the absence of double bones (for instance two right humeri), the size and tenuity of long bones, some bones with not fused epiphysis, and a milk tooth still in place in its mandibular alveolus. For sure, these bones are not compatible with the skull of an adult male ibex discovered near the main fireplace by Antonini (1986) as pictured in Figure 1, nor with the skull of an old female ibex found in Sala delle Ossa by Coltorti & Sala (1978). Nevertheless, our taphonomic observations lead to the doubt that the two groups of bones, as we found them in Site 8A, rested in situ since the time they were consumed probably by prehistoric troglodytes. It is difficult to envision that Epigravettian hunters after eating one or more ibex animals around a fireplace in this cozy hypogal environment, would have eventually disposed of the bones in that corner of the cave. It is more likely that the bones were originally scattered all over the place and then, in recent times, spelunkers inspecting the cave floor in search of collectable finds, picked up these bones, even centimeter-sized fragments, and just tossed them in that corner because they were of scarce interest (i.e., in collector’s jargon, they were
not *keepers*). The same cannot be said for bone n. 17 (Fig. 9B), which was found isolated beneath the western wall of the tunnel in Site 8B (see Fig. 5A for location). This bone fragment is the diaphysis of a bovid tibia, with a surface markedly more weathered than the bones in Site 8A, suggesting that it does not belong to the same juvenile animal. Moreover, this bone has been longitudinally fractured just as almost all long bones of Site 8A but a closer inspection suggests that the fracture was subsequently retouched to create a sharp curved edge terminating in a point. Similar bone artifacts were found, for example, in the late Epigravettian (13,000-13,500 yr cal. BP) site of Riparo Dalmeri (Trentino, southern Alps), which were typologically classified as “point with distal finishing PFD3” by Bazzanella (2005).

**Chert artifacts**

The chert artifacts found in Sala del Fuoco (Fig. 10) and currently preserved in the archive of the Archeological Superintendence of Marche in Ancona (Forestalp, 1987), consist of three simple-knapping, unretouched blades, and a well-crafted point on blade support with deep simple retouching partially covering the apex at the left distal end of the dorsal side. This point can be classified as type P2 (deep point) tending to type F3 (flat face point) according to the systematic classification of Laplace (1964). These artifacts are all made up of a high-quality, isotropic red radiolarian chert found in place as nodules and slabs do not exhibit the kind of texture, such as growth laminations perpendicular to the flat surface, which is typical of stalactitic curtains, but rather thin laminations parallel to surface as they can be found in stalagmites, flowstones, or speleothemic floor crusts (e.g., Fig. 7C). Therefore, we inferred that the piled-up slabs were derived from the floor and not from the ceiling of the cave. In fact, the cave floor immediately in front of the slab mound, which is made up of uncremented alluvial and/or fine breccia sediment, is flat and free of speleothemic crust (Fig. 11). In short, we speculated that an original thin speleothemic floor crust has been broken up into pieces, which were then tossed on the side to form a mound, an action that can be performed only by humans.

The question now arises why presumably Paleolithic ibex hunters, would have engaged in the labor of removing a hard calcite crust from the floor of this

The slab pile

The southwestern side of Sala del Fuoco is bound by boulder breccia deposits, which testify to a history of collapses of the vaults probably caused by strong earthquakes (Fig. 5A). These boulder breccias are a common feature in all large rooms of the Grotta del Fiume-Grotta Grande del Vento cave complex (Bocchini & Coltorti, 1990), including Sala delle Ossa, which is adjacent to Sala del Fuoco. However, in a small area on the northern end of Sala del Fuoco's boulder breccia (Site 4 in Fig. 5A; see panoramic view in Fig. 11), we noticed an unusual deposit of hand-size, 1 to 3 cm-thick slabs made up of speleothemic calcite, covering an area of about 1.5 x 1.5 m. The slabs do not exhibit the kind of texture, such as growth laminations perpendicular to the flat surface, layers in the pink pelagic limestones of the Eocene R4 member of the Scaglia Rossa Formation in the Umbria-Marche Apennines (Montanari et al., 1989), but also as pebbles and larger isolated cobbles in calcareous gravels making up Quaternary fluvial terraces throughout this region.

These four chert artifacts represent *per se* a non-diagnostic, very small sample of Upper Paleolithic lithic industry. Abundant and diversified chert artifacts were found in a few archeological digs of Early Epigravettian workshops in a restricted area of the eastern foothills of the central Marche ridge, namely at Monte di Pietra near Arcevia, some 10 km NW of Frasassi, Fosso Mergaoni near Serra San Quirico (21,400 yr cal. BP), about 8 km NE of Frasassi, Baracche (18,200 yr cal. BP), and Madonna dell'Ospedale near Cingoli, some 20 km E of Frasassi (e.g., Broglio et al., 2005; Silvestrini et al., 2008, and references therein). These few archeological sites suggest brief periods of occupation by probably nomadic hunter-gatherers communities, which were finalized to the production and export of lithic artifacts on blade support (Silvestrini & Peresani 2007). Therefore, we can assess that the chert artifacts found in Sala del Fuoco were crafted in the period between 17,000 and 16,000 years BP, as radiocarbon dating of charcoal and ibex bones found associated with them would suggest, and that they testify a rare Epigravettian lithic industry in this area of the northeastern Apennines.

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deep cave. A first hypothesis coming to our mind was that these people wanted to dig a ditch in the loose sediment sealed under the hard calcitic crust, perhaps to create a grave to bury a corpse. In order to test this hypothesis in a non-destructive manner, we carried out a ground penetrating radar (GPR) survey over the flat floor immediately in front of the slab mound (see Site 6 in Fig. 5 for location) covering an area of about 8 x 6 m (Fig. 12A). A similar GPR survey was performed by one of us (M. M.) in an abandoned medieval Jewish cemetery in Ancona, now a city park known as Campo degli Ebrei (Mainiero, 2003). The job, which was commissioned by the City of Ancona on behalf of the city’s Jewish community for the reclamation of this historical site and the eventual transposition of human remains, consisted in utilizing the sensitive GPR instrument to localize underground anomalies in the reflected radar signals, which could be interpreted as reworked volumes of sediment, coffins, or skeletons.

While the GPR investigation in the Jewish cemetery was successfully accomplished, our search for a grave or a buried skeleton in Sala del Fuoco, which would have produced anomalies such as hyperbolic reflections in the size range of 70 to 150 cm, did not give positive results. Nevertheless, the survey gave some interesting information about the structure and composition of the alluvial deposit making up the floor of Sala del Fuoco down to a subsurface depth of 3 m. The longitudinal sections parallel to the front of the slab mound (Fig. 12B to E) reveal a fairly homogeneous sedimentary body but with a gradual decrease with depth of the strength of the reflected signals. This is probably due to variations in the composition/texture of the sediment (sand vs. clay), and/or variations in the water content with depth. In lines L01 and L02 (Fig. 12B and C, respectively), an irregular body some 5 m wide with strong reflected signals appears at a depth of about 2 m. We interpret this anomaly as the buried toe of the boulder breccia onto which the slab mound rests, which slopes up to the W for a meter or so above ground level (Fig. 11). At a distance of about 3 m from the edge of the slab pile, this anomaly is no longer present. Similar signals are present at about the same depth in the transverse lines T01 to T09 (Fig. 12G to K), which could also be interpreted as representing the toe of the same buried boulder breccia. Finally, all the transverse lines except for T01 (i.e., T02 to T13 in Fig. 12F to M) reveal an inclined signal starting at a depth of about 1.5 m, at a distance of 3 m from the edge of the slab pile, and with a rake toward NW, which may represent an angular unconformity within the alluvial deposit. A similar anomaly is also present in longitudinal line L04 (Fig. 12D).

The negative result of our GPR survey, which was aimed at identifying a grave or a buried skeleton, did not invalidate the original inference that the floor crust has been broken up into pieces by human hands, and that the broken slabs were then stacked up on the side to form a mound. As an alternative explanation, we are herein proposing the hypothesis
that the slab pile may represent a burial structure, i.e., a sort of earthen mound (e.g., Arias, 2009). In this case, a corpse would have been laid down in a recess of the boulder breccia and then covered up with the slabs stripped out of the speleothemic floor crust. Needless to say, this hypothesis can only be tested by carrying out a proper archeological dig, which implies the removal of the slabs from the mound, an operation that would require the permission and supervision of the regional Archeological Superintendence.

Fig. 12. A) Grid of the ground penetrating radar survey carried out on the flat floor in front of the slab pile on Site 6 (see Fig. 5A for location); B to M) Longitudinal (L01 to L06) and transverse (T01 to T13) sounding profiles.
DISCUSSION AND CONCLUSION

Combined radiocarbon dating of charcoal remains from three fireplaces in Sala del Fuoco of the Grotta del Fiume Cave indicates that this hypogean environment was visited by humans some 16,201 ± 198 yr BP, while a fourth fireplace in the same room yielded a slightly older age of 16,779 ± 221 yr cal. BP. Therefore, these dates with their 2σ analytical uncertainty suggest that this cave room was visited by man through a period between about 17,000 and 16,000 years ago. In the climatic event-stratigraphy model of Rasmussen et al. (2014), this millennium falls within the cold Glacial Stadial 2.1a (GS-2.1a) in the Last Glacial period, practically corresponding to the so-called Oldest Dryas event, or the Gschnitz Stadial in the later part of the European Würm glaciation, which shortly followed the maximum extent of continental glaciers (LMG) in the northern hemisphere between 26,000 and 19,000 years ago. In the Greenland oxygen isotope record of ice cores GRIP, GISP2, and NGRIP, the cold event GS-2.1a ends with a sharp positive δ18O shift at 14,700 years BP to mark the beginning of the temperate (warmer) Glacial Interstadial 1e (GI-1e; Rasmussen et al., 2014), which is equivalent to the Bolling climate oscillation. During the GS-2.1a period, the Umbria-Marche region was not glaciated at altitudes lower than 1,500 m asl. Presumably it was a time of generalized periglacial, dry and cold environmental conditions with scarce arboreal coverage (e.g., Ravazzi et al., 2007), which, in any case, was preferentially localized along aggrading river valleys, streams, and wetlands. At present, there are no palynological data available in literature that could give some insight on the paleoenvironmental conditions in the Frasassi area during the millennium 17-16 ka. Eventually, a paleobotanical analysis of the charcoal remains in Sala del Fuoco may provide some discernment on the type of wood that was used to light the fires in this cave so to give an idea of the type of vegetation growing in the Frasassi gorge at that time.

Nevertheless, the analysis of a stalactite from Grotta Grande del Vento (sample NIA-1 in Kudielka et al., 2019), which yielded a high resolution oxygen and carbon stable isotope record calibrated with several U-Th dates, indicates that a sharp negative δ18O shift occurs in the millennium 18-17 ka, thus a thousand years before the period of human frequentation in Sala del Fuoco, but also some 2-3 thousand years before a similar sharp δ18O shift recorded at ~15 ka in the Greenland ice cores (Rasmussen et al., 2014, and references therein). The apparent diachronism of this sharp climate change as recorded in speleothems, has been observed in other caves around the world. In the Soreq Cave in Israel, for instance, the δ18O negative shift is recorded in the millennium 19-18 ka (Kudielka et al., 2019, and references therein), whereas in the Hulu Cave in China, a similar isotopic shift was dated at about 15-14 ka (Wang et al., 2010), thus consistent with the Greenland ice cores. An eventual high resolution integrated analysis of the stump of the fallen stalagmite SDF-fstg83 described above (see Fig. 11), which would combine U-Th dating and clumped isotope analysis, will give a precise date for the climate change and a better determination of the environmental conditions (i.e., ambience temperature) of the cave when it was frequented by man in the millennium 17-16 ka.

Whatever the climatic-environmental condition in the Frasassi Gorge was in the millennium 17-16 ka, the cozy Sala del Fuoco room must have provided an exclusive environment for the few Epigravettian hunters who used it for some special occasion rather than daily residential purposes. For one thing, the vast flat-bottomed Sala del Fuoco room opens up in the underground at the end of a 30 m long, curved cuniculus (see Site 9B in Fig. 5A), which would have blocked any thread of daylight, leaving the room in absolute darkness. Therefore, the hunters must have brought in dry wood and the supplies necessary to light a main bonfire in a well-ventilated spot at the foot of an imposing giant stalagmite (the Totem). But in order to reach the fireplace, they had to walk 40 m from Site 8A (the inner end of the entrance tunnel) to Site 1, watching their step, and so they must have had some sort of torches to see where they were putting their feet in that dark, spooky underground environment. The charcoal traces found in sites 1A and 1B may represent the leftovers of these torches and they yielded radiocarbon dates indistinguishable from the main fireplace and the juvenile ibex of Site 8A. It remains unsure whether the age of the fireplace in Site 1C, with its radiocarbon date several centuries older than the mean age of the other fireplaces, hides an accuracy error or possibly that the dry wood used to light that particular fire was much older than the wood used for the other fires.

Many decorated deep caves in northern Spain and southern France, which required fire lighting to illuminate the otherwise pitch-black environment, witnessed groups of Upper Paleolithic people repeatedly gathering in there to perform, besides parietal art, all sorts of hypothetical social, cultural, or religious activities and rituals leaving behind remains of combustion, stone stacking structures, and intentionally deposited materials and objects (e.g., Medina-Alcaide et al., 2018, and references therein). Contextually, our undecorated but equally deep and dark Sala del Fuoco cavern only exhibits evidence for a few fire lighting, a few bones of ibex probably eaten on site by man, and four lithic artifacts crafted from local chert. In addition, a seemingly unnatural pile of speleothem slabs may represent a stacking structure. The questions that ask for rational answers are now: 1) how many people have used Sala del Fuoco in the millennium 17-16 ka or possibly in the four centuries between 16.4 and 16.0 thousand years ago? 2) how many times?, and 3) for what reason? Judging from the three ibex individuals identified so far in this cave, i.e., the old female specimen reported by Coltorti & Sala (1976) in Sala delle Ossa, the adult male set on the tip of a stalagmite near the main fireplace as reported by Antonini (1986), and the juvenile specimen here identified in the bone deposit of Site 8A, leads us to speculate that a couple of dozen or so hunters would have been able to eat all that game.
meat (i.e., about 200 kg in total). However, it may well be that these three ibex specimens were killed in three different hunting parties by smaller hunting bands more likely composed of a few individuals. Shedding some light on the question of how many times small groups of Epigravettian hunters have visited this cave would require an archeological excavation of the main fireplace in Site 1 and also the fireplace of Site 1C, which can be carried out only by specialized personnel with the required by-law authorization of the regional Archeological Superintendence.

As for the question of why these hunters ventured in this cave bringing in big hunted game and wood for lighting fires instead of bringing the catch of the day to their settlement, whether a camp or a hut village down in the valley, or a more comfortable and easy-to-reach cave in the crags of the Frasassi Gorge, the most plausible explanation is that they gathered there to perform some sort of a ritual or ceremony. Not excluding that it may have been like a fraternity party for an initiation to adulthood or leadership, one possibility is that small groups of hunters were gathering there to worship either a deity or a dead fellow. A funeral worship scenario is suggested by a seemingly unnatural deposit of speleothem slabs piled up in Site 4 among collapsed boulders, which may represent a burial earthen mound. However, the only way to test this hypothesis will be to remove the slabs, an operation that requires, once again, specialized archeological personnel and the authorization of the regional Archeological Superintendence.

In conclusion, our non-invasive environmental study of the Sala del Fuoco cavern and the geochronological assessment via radiocarbon AMS dating of charcoal remains and ibex bones found therein, lead us to the hypothesis that this hypogeal space was seldom visited, in the late Upper Paleolithic (17-16 ka), by small groups of hunters for ritual and possibly funeral purposes. Further on-site research, which implies archeological digs by specialized personnel with the by-law authorization of the regional Archeological Superintendence, will hopefully verify our hypothesis and in any case shed more light on the Epigravettian culture in this region of central Italy.

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Cosmogenic nuclide dating of cave sediments in the Eastern Alps and implications for erosion rates

Philipp Häuselmann1,2*, Lukas Plan3, Peter Pointner4, and Markus Fiebig2

1Swiss Institute for Speleology and Karst Studies SISKA, Serre 68, 2301 La Chaux-de-Fonds, Switzerland
2Institute for Applied Geology, University of Life Sciences BOKU, Peter Jordan Strasse 82, 1190 Wien, Austria
3Karst and Cave Group, Natural History Museum Vienna, Museumsplatz 1/10 A-1070 Wien, Austria
4Society of Speleology of Salzburg, Schloss Hellbrunn – Objekt 9, A-5020 Salzburg, Austria

Abstract: Karstic caves are created by water eroding and corroding rocks that can be dissolved. Since both the spring areas of caves (normally at the valley bottom) as well as the recharge is controlled by superficial processes, the morphology of the cave bears strong links to these influences. Lowering of local base levels promotes the development of horizontal phreatic cave passages at progressively lower elevations, resulting in the formation of multi-level karst systems. Upon the next lowering of base level, these upper systems become fossilized, and sediment trapped within them may remain preserved for millions of years. Dating these sediments gives clues regarding the time when the passages were last active, and thus may yield age information for old valley floors. The present paper presents cosmogenic nuclide datings of twelve samples from eight caves in the central part of the Northern Calcareous Alps of Austria. Besides three samples that gave no results, most of the obtained ages are at the Mio-Pliocene boundary or within the Pliocene, as was expected before sampling. No multi-level caves could be sampled at different elevations, thus, the obtained valley deepening rates are averages between the age of sediment deposition and the present-day valley floor. However, the valley deepening rates of 0.12 to 0.21 km/Ma are in accordance to previous findings and corroborate a comparatively slow evolution of base level lowering in the Eastern Alps compared to the fast (Late Quaternary) evolution in the Central and Western Alps.

Keywords: Eastern Alps, cosmogenic dating, caves, valley incision, landscape evolution

INTRODUCTION

Speleogenesis and surface landscape development in mountain ranges is the result of interplay between tectonic uplift and climate-controlled erosion. Quantifying the rate at which landscape-modelling processes acted in the past requires the accurate dating of geomorphic markers. In rapidly eroding landscapes – especially if they have been glaciated – these markers are often removed by subsequent erosion, so that records are missing for the last few million years. Landscape evolution in the European Alps has been a major topic of research for Earth scientists during the past decades (Kuhlemann, 2000; Frisch et al., 2008). The effects of landscape modeling processes are quantified using estimated sediment budgets, fission track analysis (Cederbohm et al., 2004), and the dating of geomorphic markers like fluvial terraces, glacial moraines, and erosion surfaces.

Caves can provide additional clues for understanding landscape development. In karst regions, gradual lowering of valley floors and local base levels by river incision or glacial erosion promotes the development of horizontal phreatic cave passages at progressively lower elevations, resulting in the formation of multi-level karst systems (Audra et al., 2006; Columbu et al., 2015). Sediments that have been washed into these caves may be preserved from on-going erosion.

Dating sediments from various cave levels can provide information about the pace of landscape development, and in particular, valley incision rates, provided that the sediments are in a primary position and can be related to the development of the cave (Granger & Muzikar, 2001). The minimum age of a cave can be determined by dating the sediments it contains, which are younger than the cave itself. The most widely used techniques are U-Th disequilibrium dating, paleomagnetic analysis, and burial age dating.
on cosmogenic nuclides. Applications in the field of cave paleontology is also possible, particularly for dating sites of Middle Pleistocene to Pliocene age. U-Th dating can seriously underestimate the age of the caves, because speleothems can grow a long time after the cave formation, and foremost, the method is limited to ca. 0.5-0.7 Ma (Stock et al., 2005a). Paleomagnetic dating requires correlation with the global reversal chronology (e.g., Häuselmann et al., 2015), but in caves, a continuous stratigraphy is rarely available.

The burial age method involves the measurement of two isotopes (26Al and 10Be) that are produced by cosmic radiation in quartz near the surface prior to burial. 26Al and 10Be accumulate at a ratio of about 6.8:1 (Balco et al., 2013) in quartz grains with a rate of a few atoms per gram of quartz per year. Sufficiently deep burial (more than 10 m) of such quartz-rich sediment in a cave assures shielding from further cosmic rays. After burial the 26Al and 10Be concentrations in the sample are only affected by their relative decay resulting in a decrease in the 26Al/10Be ratio. This ratio measured can be used to derive a burial age (Gosse & Phillips, 2001; Granger & Muzikar, 2001). The current upper limit for measurement of the 26Al and 10Be isotope pair is around 5 Ma. A prerequisite of the burial dating technique is that samples have been exposed long enough to cosmic rays and accumulated sufficient cosmogenic nuclides prior to burial. Unfortunately this cannot be determined a priori in the field. The burial dating method was already successfully applied in the Western Alps (Siebenhengste, west-central Switzerland), where the incision rate of the valleys was estimated to have been 0.14 mm/a during the Pleistocene and 0.28 mm/a during the Middle (and Upper) Pliocene (Häuselmann et al., 2007).

With the exception of a study on non-glaciated parts of the Eastern Alps (Wagner et al., 2010), there were no burial age dating results so far in the Eastern Alps. Here we present age dating for a range of caves in the Northern Calcareous Alps, a region which was glaciated during the Pleistocene and from which no hard age dating regarding the time of cave formation has been given so far.

PREVIOUS STUDIES ON LANDSCAPE EVOLUTION IN THE EASTERN ALPS

Data for the Eastern Alps (Kuhlemann et al., 2001) indicate a slow erosion rate in the Miocene (0.2 mm), followed by an increase in the Pliocene and Pleistocene. Szekely (2003) used digital elevation models to quantify uplift and erosion rates. He concluded that uplift was in the order of 0.13 mm/a in the Pliocene and 0.28 mm/a in the Quaternary, thus much slower than the previous studies. Fission track data from the Central Alps (Bernet et al., 2001) assume a steady-state exhumation of 0.4 to 0.7 mm/a for the last 15 Ma.

The Northern Calcareous Alps (NCA) provide an ideal application for landscape evolution studies based on cave sediments because the caves show a clear vertical distribution as they form levels at certain altitudes. Attempts have been made to empirically link this distribution pattern to the geomorphic history of the Alps, but numerical ages for the caves could not yet be determined. Estimations based on data other than caves indicate slow valley incision rates during the Miocene and an increase during the Pliocene and Pleistocene (Kuhlemann et al., 2001).

While the western part of the NCA lack plateau morphologies, the eastern part of the NCA is characterized by huge (up to 1,000 km²) karst plateau systems developed at elevations between roughly 1,600-2,500 m a.s.l. (Frisch et al., 2001). The NCA consist of a sedimentary succession ranging from Permio-Triassic to Eocene times. The plateaus developed mainly on an often more than 2 km thick sequence of Triassic carbonates dominated by the Upper Triassic Dachstein limestone.

During the past century, two competing theories emerged on the genesis of these plateaus: (1) the idea of the Rax Landscape, a one-phased uniform peneplaned paleosurface which was later dismembered and differentially uplifted (Lichtenecker, 1924, 1926) and (2) a polycyclic piedmont formation (Augenstein Landscape) with several periods of tectonic quiescence in which planation surfaces of limited extent formed, interrupted by repeated phases of uplift (Winkler-Hermaden, 1957; Langenscheidt, 1986).

The controversy was lifted by Frisch et al. (2001, 2002) who summarized the evolution as follows: the karst plateaus are relics of the so-called Dachstein Paleosurface that formed in late Oligocene to early Oligocene times as karst peneplains. In the Oligocene, the paleosurface subsided and was sealed by the Augenstein Formation, a terrestrial sequence of conglomerates and sandstones. The material of the Augenstein Formation is rich in polycrystalline quartzite and consist of greywacke slate, phyllite, gneiss, and carbonates. The source area of the sediments was south of the NCA in the Paleozoic Greywacke Zone which is only a narrow strip today but covered significant parts of the Austro-Alpine Crystalline during the Paleogene. Deposition of the Augenstein ended in the late Early Miocene, when E-W-trending valleys formed in the south of the NCA, cutting off sediment input from the source area (Frisch et al., 1998). Following the uplift of the NCA, the bulk of the Augenstein Formation was eroded and re-deposited in the Molasse basin north of the Alps. From Upper Miocene times (~10 Ma), the denuded paleosurface experienced uplift in several pulses, but as karstification of the thick limestones allowed the development of underground drainage, no significant surface erosion took place and the landscape was preserved (Frisch et al., 2001).

The karst massifs of the central and eastern NCA (Dachstein, Tennengebirge, Totes Gebirge, etc., Fig. 1) host several thousand caves of which some are quite huge: presently there are 35 caves where more than 10 km of passages have been mapped, of which the Schönberg-Höllensystem is the longest with 148 km (Pfarr et al., 2019). Most of the subhorizontal passages are arranged in three distinct cave levels (Hasek-Knapczyk, 1989; Fischer, 1990) which suggests that the uplift of the NCA occurred in pulses separated
by periods of tectonic inactivity (Frisch et al., 2001, 2002). The highest and therefore oldest caves are currently located at altitudes between 1,900 and 2,500 m a.s.l. (ruin cave level or Ruinenhöhlen), which, according to Frisch et al. (2001), could have formed already during the formation of the Dachstein Surface in upper Eocene to lower Oligocene. The largest system of caves at altitudes of 1,400-1,800 m a.s.l. (giant cave level or Riesenhöhlen) have formed after the sedimentation and removal of the Augenstein Formation in an early stage of the final uplift period of the NCA in Upper Miocene. The third and youngest level is situated close to modern valley floors and is partly still active today (spring cave level or Quellhöhlen).

Based on karst morphologic studies carried out in the Tennengebirge, Audra et al. (2002) assigned a Miocene age to the uppermost Ruinenhöhlen level, while the Riesenhöhlen level was thought to have formed during Upper Miocene to Pliocene times.

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**Fig. 1. Simplified map of the Northern Calcareous Alps (NCA) and adjacent areas. Lower left: situation in Austria. GW – Greywacke zone, PG – Paleozoic of Graz, GN – Gurktal nappe (from Frisch et al., 2001, redrawn). Ds: Dachstein; Hk: Hochkönig; Hs: Hochschwab.**

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**SITES AND SAMPLES**

A total of 15 samples were collected from caves situated in the Leoganger Steinberge, Hagenengebirge, Untersberg, Tennengebirge, Dachstein, and Totes Gebirge karst massifs (Table 1 and Fig. 2). Reviews of the areas concerning karst and caves can be found in Spötl et al. (2016). Samples collected from Totes Gebirge contained only very small amounts of quartz and proved to be unsuitable for burial age dating, but relatively clean samples have been obtained from Dachstein and Tennengebirge. Additional fieldwork was conducted in the Hoher Göll, but no quartz-containing sediments were found.

**Dachstein**

The Dachstein plateau is the second largest karst plateau in the NCA, covering a surface of approximately 550 km². Altitudes range from 2,995 m a.s.l. (Hoher Dachstein) to 508 m a.s.l. (Hallstatt Lake). The plateau lacks organized surface drainage and is dewatered exclusively by subterranean streams.

Speleogenesis in the area is controlled by gently N-NW dipping bedding planes of the Dachstein Limestone and a complex set of faults. A particular large number of caves and cave passages have been identified on the northern edge of the plateau, which is characterized by steep slopes and vertical walls. The largest caves in the area are the Hirlatzhöhle (113 km) and the Dachstein-Mammuthöhle (67 km) both of which feed karst springs which drain into the Hallstatt Lake (Fig. 3).

Mammuthöhle shows one distinct cave level, which belongs to the Riesenhöhlen level, at 1,300-1,450 m a.s.l. Vertical shafts provide connections both to the surface and to the lower active cave levels. Most parts
of Hirlatz cave are developed between 950 and 1,500 m a.s.l. but its relationship to a distinct cave level is not clearly evident.

Allogenic cave sediments consist of well-rounded pebbles and sand layers that may be locally cemented, overlain by laminated silt and clay of glacial origin. Speleothems are very rare above these clastic sediments in Dachstein-Mammuthöhle, massive speleothems are generally older than U/Th limits, and a $^{238/235}$U equilibrium of one stalagmite suggests an age of more than 1.5 Ma (Frisch et al., 2002). In some parts, the fluvial pebbles show two distinct lithologies. At the base, a thin quartz/quartzite dominated layer occurs, overlain by dominantly carbonate material, which is present in much larger quantities. In general, the two lithologies are rarely found in direct contact inside the cave, but stratigraphical arguments suggest that the quartzite bearing sediment is the older one (removal of the Augenstein sediments from the plateau was followed by erosion of the carbonates). Both the thickness and the grain size of the quartziferous beds is higher in Mammuthöhle where pebbles with diameters of 2-4 cm are common, while in Hirlatz, quartz pebbles were only found in isolated locations and had diameters of 1-2 cm.

In the Dachstein-Mammuthöhle, all samples consisted of large quartzite pebbles which were collected from different parts of the cave, from altitudes ranging between 1,310-1,440 m a.s.l. Sample DMH1 was taken from a small passage of the
Derflinger Labyrinth (Fig. 4) situated at an altitude of 1,310 m, where the quartzite pebbles were cemented to the passage walls. This is one of the few places in the cave where a clear stratigraphic relationship can be established between the quartzite and limestone pebbles.

Samples DMH2 and DMH3 were taken from the Paläotraun passage which is part of the Mammuthöhle show cave. DMH2 was taken at an altitude of 1,360 m, at the eastern end of the Paläotraun passage, from a bed of loose, mixed quartzite and limestone pebbles and sand. Sample DMH3 was taken further along the Paläotraun Passage and consist of large quartzite pebbles that were cemented to the passage floor sculptured by scallops. The scallops are several centimeters to decimeters long and indicate westward drainage under epiphreatic conditions (Plan & Xaver, 2010).

Sample DMH4 was taken from Wassergang gallery in Alter Teil at an altitude of 1,440 m and consisted of a deposit of mixed quartzite pebbles and material from the weathering of the Augenstein formation.

The two samples from Hirlatz cave were both collected in the western part of the cave, from a passage that extends between 900-1,000 m a.s.l. and is interrupted by several large chambers. The area is often flooded during snow-melt or summer storms. Fluvial sediments consisting of rounded limestone pebbles and laminated clay are common. Sample HR1 consists of quartzite pebbles cemented by calcite, while HR2 consist of loose pebbles found on the floor of the passage.
Tennengebirge

The sampled caves are located on the central northern slope of Tennengebirge (Fig. 5), south of Scheffau am Tennengebirge and approximately 30 km south of Salzburg. The caves are situated above the Lammer valley which constitutes one of the main waterways of the area.

Based on their altitude between 1,100-1,500 m, it can be assumed that the caves formed during Upper Miocene to Pliocene. The position of the three caves sampled in the area, Schaflschacht (1,480 m a.s.l.), Quarzloch (1,100 m a.s.l.), and Gamsställe (1,120 m a.s.l.) is shown in Fig. 5.

Schaflschacht (1500 m a.s.l.) cave consists of a 10 m deep shaft which gives access to a small horizontal passage with unconsolidated rounded fluvial pebbles. Based on comparative studies with neighboring caves, the Schaflschacht clearly belongs to the Riesenhöhlen level. The sediments consist mainly of quartz pebbles with diameters of 1-2 cm (sample SFS).

Quarzloch and Gamsställe are situated relatively close to each other (0.2 km) at roughly the same altitude, corresponding to the “Bergerhöhlen-Niveau” of Audra et al. (2006) who suggested a Pliocene age. Similar sediments were found in both caves, consisting of interbedded medium and coarse sands and red clays (Fig. 6). Clay is less common in the Quarzloch sediments, but in Gamsställe, the two lithologies form alternating beds with cycle thicknesses of 20-30 cm. Following sedimentary structures, it might be possible that the sediments could have been washed in during glaciations, however there is no absolute proof, and therefore the sediment was sampled. A potential problem with the sediments from Quarzloch is that the sampled site is located relatively close to the surface (about 10 m away from the cave entrance and overlain by about 10 m of limestone) so post-burial production could have a significant influence on the age of the sample. Given the fact that Quarzloch and Gamsställe (which is deeper than 10 m) are situated close to each other and contain similar sediments, it might be possible to estimate the influence of post-burial cosmogenic nuclide production by comparing the age of the two samples.

Lamprechtsofen

The cave is located in the Leoganger Steinberge, between the villages Lofer and Saalfelden. The main entrance opens only few meters above the floor of the river Saalach, at an altitude of 660 m a.s.l. (Fig. 7). The sample was taken from the lowermost cave level near the spring, which represents the main entrance. Compared to the extensive cave system, the collected pebbles were deposited quite close to the entrance, but nevertheless several hundred meters away from it. The collected grains are noticeably big and reach cobble-size. Fine sediments are lacking. So it is likely, that these sediments are reworked from higher altitudes by the cave river named “Steinbach”. It is not excluded that the sampled sediment was washed into the cave from the entrance below, however the presence of several passage jumps makes this possibility rather improbable.
Tantalhöhle
With its 35 km, Tantalhöhle is the longest cave in the Hagengebirge. Its entrance is located within the southern rock walls at 1,710 m a.s.l., so this cave belongs to the Riesenhöhlen level (Fig. 8). The depth of the cave is 440 m, with the entrance as the highest point. The sediment sample was taken from the Sandschluf, approximately 1.5 km inside the mountain.

Normally, clayey-silty sediments dominate within Tantalhöhle. Sandschluf represents a prominent narrow passage where the grain size increases up to coarse sand. The sample was taken from the upper layer of the sediment. Due to lack of a sediment profile, there is no information about the sediment content of deeper layers at this location. Following the local cavers, the sediment was washed in from the surface in a later stage of speleogenesis, but affected by a later erosional phase. This means that the sediment could have been deposited before the last activity of the passage.

Fürstenbrunner Quellhöhle
The cave is situated in Untersberg, south of Salzburg near the Fürstenbrunn village (Fig. 9). Situated at 600 m a.s.l. and 120 m above the valley, the cave is the biggest karst spring of this massif. Its water is captured as drinking water supply for Salzburg. The cave is developed in Dachsteinkalk, has a length of 3.3 km and a depth of 135 m. The lowermost level contains the river, higher parts are periodically flooded. An older, permanently dry level is developed approximately 100 m above the active parts. The sample was taken from a small passage connecting both levels, named “Brillengang”. During high discharge, backflooding can still reach the sampling point, but...
there is no current then. The sediments consist mainly of well-rounded sand grains up to coarse gravel. Silt and stones are minor components. The biggest sediment masses occur in a lateral branch and are unstratified. In Untersberg, primarily carbonates are found and quartz-containing sediments are rare. To get some burial ages from this mountain range, this sediment seemed to be the best. Still, there is a possibility that these sediments were brought into the cave via a higher entrance that is blocked now.

METHODS

In the laboratory, about 100 g of quartz were extracted and purified from bulk samples by magnetic and density separation and selective chemical dissolution. Quartz was dissolved in a 5:1 solution of concentrated HF and HNO₃ and spiked with about 0.25 mg ⁹Be. Al and Be were separated and purified by ion chromatography and selective precipitation. Precipitates were oxidized and mixed with metal powder for accelerator mass spectrometry (AMS). ¹⁰Be/⁹Be and ²⁶Al/²⁷Al nuclide ratios in the sample and procedural blanks were measured at Purdue University in West Lafayette (USA). Stable aluminium concentrations were determined by ICP-OES. The stated errors are 1σ calculated from AMS and ICP-OES uncertainties. The uncertainties in the measurement of the procedural blanks yielded ¹⁰Be/⁹Be ratios ≤⁵ x 10⁻¹⁵ and ²⁶Al/²⁷Al ratios of <0 to 13 x 10⁻¹⁵. With the exception of the relatively high Al blank (relativized by the high error of ±18 x 10⁻¹⁵), the blanks thus show that the measurements were good and the standards clean.

The isotope concentrations can also be used to infer paleo-erosion rates of the source area prior to burial of the clasts. This is accomplished by backward modeling the quantity of nuclides present prior to the burial coupled with local production rate estimates. The pre-burial ²⁶Al/¹⁰Be ratio (~6.8:1) is basically not influenced by production rate and thus elevation (Nishiizumi et al., 1989; Stock et al., 2005b) and therefore burial ages remain unaffected by altitude changes in the source area. However, the pre-burial erosion rates are based on measured isotope concentrations and elevation-dependant production rates. They are therefore only rough approximations.

RESULTS

Table 2 gives the results of the measurements. All results were normalized for a ¹⁰Be half-life of 1.39 Ma (Chmeleff et al., 2009, Korschinek et al., 2009). The uncertainties represent the analytical error only. The erosion rates are approximate, since the altitude and further shielding problems at the surface are unknown.

The first impression is that almost all the caves that were sampled yielded ages from the Pliocene and late Miocene. This, however, does not reflect the general situation: sampling was specifically concentrated on caves whose altitude above the baselevel suggested ages that were within some millions of years. Clearly young caves and clearly old “Ruinenhöhlen” caves were voluntarily omitted, with the exception of the Fürstenbrunner Quellhöhle and Lamprechtsofen, where passages of different age are present. The distribution of the ages is therefore strongly biased by sampling.

There are three samples that yielded no age. The upper age limit is given by the gradual decay of the nuclides; after some millions of years, there are almost no nuclides left to be counted, and then the
Table 2. Results of the $^{26}$Al/$^{10}$Be measurements and the inferred ages as well as erosion rates. No blank correction was applied. The altitude of catchment is an estimate used to infer nuclide production rates at the surface, which in turn gives hints about the erosion rate at the surface where

<table>
<thead>
<tr>
<th>Cave</th>
<th>Sample</th>
<th>Altitude catchment</th>
<th>Altitude in cave</th>
<th>$^{26}$Al (10$^3$ at/g)</th>
<th>$^{10}$Be (10$^3$ at/g)</th>
<th>$^{26}$Al/$^{10}$Be Burial age (Ma)</th>
<th>Inh. Erosion</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dachstein</td>
<td>DMH1</td>
<td>1,700</td>
<td>1,310</td>
<td>5.3 ± 4.6</td>
<td>12.4 ± 1.25</td>
<td>0.43 ± 0.37</td>
<td>5.75 ± 1.31</td>
</tr>
<tr>
<td></td>
<td>DMH2</td>
<td>1,700</td>
<td>1,360</td>
<td>7.1 ± 4</td>
<td>8.6 ± 0.95</td>
<td>0.83 ± 0.47</td>
<td>4.37 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>DMH3</td>
<td>1,700</td>
<td>1,400</td>
<td>0 ± 0</td>
<td>18.4 ± 1.67</td>
<td>0 ± 0</td>
<td>beyond age limit</td>
</tr>
<tr>
<td></td>
<td>DMH4</td>
<td>1,700</td>
<td>1,430</td>
<td>0.2 ± 0.2</td>
<td>10.2 ± 0.93</td>
<td>0 ± 0</td>
<td>beyond age limit</td>
</tr>
<tr>
<td>Hirlatz</td>
<td>HR</td>
<td>1,700</td>
<td>~1,000</td>
<td>38 ± 10</td>
<td>68.2 ± 2.18</td>
<td>0.55 ± 0.15</td>
<td>5.16 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>HR2</td>
<td>1,700</td>
<td>1,060</td>
<td>21 ± 9</td>
<td>79.4 ± 3.29</td>
<td>0.27 ± 0.12</td>
<td>6.6 ± 0.72</td>
</tr>
<tr>
<td></td>
<td>Schaafsacht</td>
<td>SFS</td>
<td>2,100</td>
<td>1,480</td>
<td>63.2 ± 2.62</td>
<td>0.27 ± 0.18</td>
<td>6.65 ± 0.95</td>
</tr>
<tr>
<td></td>
<td>Quarzloch</td>
<td>QL</td>
<td>1,100</td>
<td>1,120</td>
<td>6.89 ± 1.24</td>
<td>0 ± 0</td>
<td>beyond age limit</td>
</tr>
<tr>
<td></td>
<td>Gamsställe</td>
<td>GST</td>
<td>1,100</td>
<td>1,120</td>
<td>12.6 ± 2.52</td>
<td>5.11 ± 3.58</td>
<td>0.89 ± 1.13</td>
</tr>
<tr>
<td></td>
<td>Tantalhöhle</td>
<td>TTH</td>
<td>2,000</td>
<td>1,500</td>
<td>496 ± 35.1</td>
<td>0.65 ± 0.17</td>
<td>4.67 ± 0.49</td>
</tr>
<tr>
<td></td>
<td>Lamprechtsofen</td>
<td>LPH</td>
<td>2,000</td>
<td>670</td>
<td>23.1 ± 1.87</td>
<td>2.04 ± 0.37</td>
<td>2.49 ± 0.35</td>
</tr>
<tr>
<td></td>
<td>Fürstenb. Q.</td>
<td>FBQ</td>
<td>1,500</td>
<td>700</td>
<td>17.8 ± 3.56</td>
<td>1.7 ± 2.02</td>
<td>2.87 ± 1.56</td>
</tr>
</tbody>
</table>

age calculations do not get a meaningful result. This is the case for samples DMH3 and DMH4 from Dachstein-Mammuthöhle. There, no more $^{26}$Al counts could be recorded, and thus an age calculation is no more possible. However, the presence of $^{10}$Be in these three samples shows that the sediment was at the surface once. A good guess on the age of these samples would be a deposition underground that happened in the Upper Miocene (5-10 Ma ago). After 10 Ma, all $^{10}$Be would have been decayed. Theoretically, it would be possible to estimate a minimum burial age, taking into account the surface production rate and the average residence time at the surface (taken from other samples with known age). However, since both are only gross estimations, and DMH1 and 2 gave old ages, we think it is misleading to indicate a minimum burial age.

A special case is the Quarzloch, which contains $^{10}$Be, but no $^{26}$Al. The first assumption is that this sediment had the same fate as DMH3 and DMH4. However, the cave is rather short and the sediment deposited is close to the surface, so that the interpretation is that the few nuclides present in the sample were rather initiated by irradiation from the surface.

Two samples present ages that are above the commonly assumed upper age limit of the method. Sample HR2 from Hirlatz cave as well as SFS from Schaafsacht show ages of 6.6 Ma. In HR2, however, both the $^{26}/^{27}$Al ratio measured ($2\times10^{-15}$) as well as the absolute $^{26}$Al numbers ($2\times10^3$) and the back-calculated initial isotope ratio are well within a reasonable range. We assume that although the age calculated is high, it is still reasonable. The SFS sample is a bit different in that the $^{26}/^{27}$Al ratio measured is much lower ($3\times10^{-13}$), and the final error is larger.

**EXPECTATIONS ON RELATIVE CHRONOLOGY**

Following Frisch et al. (2008) as well as Audra et al. (2002), we expected an Eocene to Miocene age for the ruin level caves, an upper Miocene age for the giant level caves, a Pliocene age for the “Bergerhöhlen” level found in Tennengebirge at an altitude of 900-1100 m asl, and a Plio-Pleistocene age for the Quellhöhlen level.

This translates into the following ages: The DMH samples, SFS and Tantalhöhle are expected to be Upper Miocene, the Hirlatz samples rather Plio-Pleistocene, Quarzloch and Gamsställe Pliocene, and Lamprechtsofen and FBQ clearly Plio-Pleistocene again. From in-cave stratigraphy, DMH is expected to be old also. Tantalhöhle is, from the stratigraphic position, expected to give the age of the last flooding of the passage before its fossilization, whereas the Hirlatz samples might be rather young (still within reach of the floods). Both Lamprechtsofen and FBQ might be young, but they were sampled at locations where this possibility should be minimized. The sediments of Quarzloch and Gamsställe are expected to be contemporaneous, since they are very comparable in facies and grain size distribution, however their rather low position make it possible that they were deposited during the Pleistocene glaciations.

**DISCUSSION**

There is always the question whether the obtained ages mean anything: sand and cobbles are mobile, and floods may well dislocate old sand deposits, mix them, and deposit in a much younger cave section, so that any age dating is prone to be erroneous.

Indeed, polycyclic hydrologic activity is common in Alpine caves. Often, existing galleries are reused by younger vadose waters from intersecting canyons. Another phenomenon causing sediment (re)deposition is backflooding during the Pleistocene, when the valleys were occupied by partly more than 1 km of ice.

The lack of extraordinary young ages at high elevations (except from Gamsställe) suggests that among the analyzed samples there was no surface material that was washed into a preexisting gallery at a later stage. However, the samples from Fürstenbrunner Quellhöhle and Lamprechtsofen are surprisingly old despite their low elevation and do
not fit into the picture. We interpret them as old cave sediments that were eroded from higher locations and redeposited at the sample location relatively lately.

With the exception of the latter two, the explanation fits to the experience from the Siebenhengste example (west-central Switzerland; Häuselmann et al., 2007): all the young passages contained only young sediments, whereas old passages contained old and young sediments. This means that deposition occurs over the whole age span of a cave, whereas remobilization results in a washout of the sediments to the surface. This process is currently observed in Höllloch cave (Switzerland), where old sediments were mobilized by the huge 2005 flood (320 m above normal lowstand, 100 m above historic highstands) and are now continually transported towards the entrance of the cave.

The DMH samples with their lower Pliocene age are a bit younger than expected, but still within the possible range, especially when considering the (for Riesenhöhlen) rather low altitude.

The Hirlatz ages are very high, both regarding the sample position close to the active parts, and the absolute altitude. It is likely that these again are old, reworked sediments that were brought into lower passages by exceptional floods.

The Schafsacht and Tantalhöhle ages again are in good accordance, but the Gamsställe shows an age that is too young. The sample, which, as said above, was possibly been washed in during glaciations, is now clearly Quaternary.

If we assume that the sediments were washed into their position from the surface at the time indicated by their age (that is, they were not reworked from an older cave level), and again assuming that they were deposited at approximately the base level, as indicated in several cases by passage morphology, we can tentatively calculate a valley deepening rate. Table 3 shows that deepening rates that can be used are between 0.12 to 0.21 km/Ma, and that the older ages yield lower deepening rates than the younger ones.

**Comparison with previous studies**

While age dating with the U/Th method on stalagmites (in connection with climate research) is widespread, cosmogenic age dating is rare. In Austria, a first attempt on sediments from Eisriesenwelt yielded no results (Frisch et al., 2002), and only Wagner et al. (2010) presented coherent and logical data on the incision of the Mur river north of Graz in an unglaciated area being 0.12 km/Ma at average for the last 4 Ma. Comparison with other published cosmogenic ages in the Alps (Häuselmann et al., 2007) shows a comparable age range, with valley deepening rates in this glaciated part of the Alps ranging from 0.12 km/Ma (Pliocene) to rapid 1.2 km/Ma after the mid-Pleistocene transition around 1 Ma ago.

Meyer et al. (2009) dated speleothems in a cave in the Allgäu Mountains (NCA) to 2.16 to 2.12 Ma by U/Pb. The cave's altitude is at 2,500 m a.s.l. They concluded that uplift and erosion rate in that part of the NCA amounts to roughly 0.75 and 0.5 km/Ma, respectively.

Frisch et al. (2008), in the paper on the geomorphic evolution of the Eastern Alps, concluded that the averaged Mio-Pliocene erosion rate was increasing from 0.1 to 0.2 km/Ma, and to 0.3 km/Ma in the Quaternary.

A regional cave study in Kraushöhle (De Waele et al., 2009) indicates, however with a large error, a minimal uplift rate of 0.44 km/Ma in that area. This data points indeed to an accelerated deepening rate early in the Quaternary.

Comparing the above deepening rates with our results, we have first to note that in contrast to the data published by Häuselmann et al. (2007), the rates obtained here are the total deepening rates from sample deposition to present-day status. Notwithstanding this averaged rate, the valley deepening rates presented here are in good accordance with both the conclusions of Frisch et al. (2008) as well as the results for the older speleogenetic phases of the Siebenhengste before the mid-Pleistocene transition.

Regarding the geomorphic history of the Northern Calcareous Alps, our results also confirm reasonably well the evolution described by Frisch et al. (2002) and Audra et al. (2006) as well as Kuhlemann et al. (2001). Upper Miocene to Lower Pliocene ages of the giant cave level of the NCA are therefore reasonable.

<table>
<thead>
<tr>
<th>Cave</th>
<th>Sample</th>
<th>Depositional altitude</th>
<th>Present-day altitude</th>
<th>Burial age (Ma)</th>
<th>Deepening rate (m/Ma)</th>
<th>Remarks</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dachstein</td>
<td>DMH1</td>
<td>1310</td>
<td>508</td>
<td>5.75 ± 1.31</td>
<td>139</td>
<td></td>
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<tr>
<td></td>
<td>DMH2</td>
<td>1360</td>
<td>508</td>
<td>4.37 ± 0.95</td>
<td>195</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMH3</td>
<td>1400</td>
<td>508</td>
<td>0 ± 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>DMH4</td>
<td>1430</td>
<td>508</td>
<td>0 ± 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hirlatz</td>
<td>HR</td>
<td>1000</td>
<td>508</td>
<td>5.16 ± 0.49</td>
<td>95</td>
<td>reworked?</td>
</tr>
<tr>
<td></td>
<td>HR2</td>
<td>1060</td>
<td>508</td>
<td>6.6 ± 0.72</td>
<td>84</td>
<td>reworked?</td>
</tr>
<tr>
<td>Schafsacht</td>
<td>SFS</td>
<td>1480</td>
<td>660</td>
<td>6.65 ± 0.95</td>
<td>123</td>
<td></td>
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<tr>
<td>Quarzloch</td>
<td>QL</td>
<td>1120</td>
<td>660</td>
<td>0 ± 0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gamsställe</td>
<td>GST</td>
<td>1120</td>
<td>660</td>
<td>0.59 ± 1.13</td>
<td>780</td>
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<tr>
<td>Tantalhöhle</td>
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<td>1500</td>
<td>500</td>
<td>4.67 ± 0.49</td>
<td>214</td>
<td></td>
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<tr>
<td>Lämprechtsofen</td>
<td>LPH</td>
<td>670</td>
<td>660</td>
<td>2.49 ± 0.35</td>
<td>4</td>
<td>reworked</td>
</tr>
<tr>
<td>Fürstenb. Q.</td>
<td>FBQ</td>
<td>700</td>
<td>425</td>
<td>2.87 ± 1.56</td>
<td>96</td>
<td>reworked</td>
</tr>
</tbody>
</table>

Table 3. Approximate valley deepening rates of the base levels in the respective caves. To be complete, also the cave samples that clearly were reworked are calculated.
The results of our study confirm the data from Szekely (2003) and indicate a somehow slower erosion rate than Kuhlemann et al. (2001). The data from the Central Alps (Bernet et al. 2001) are much higher than ours, but it is generally assumed that the Eastern Alps have a different (and slower) exhumation/incision history.

CONCLUSIONS

Cosmogenic dating of caves in the Northern Calcareous Alps of Austria yielded somehow mixed results. While some of the ages obtained correspond to the expectations that the Riesenhöhlen level is Upper Miocene, and Quellhöhlen level is Plio-Pleistocene (DMH, including the two samples above the age limit, Schaflschacht, and Tantalhöhle), others seem to give ages too old (Hirlatz, FBQ, Lamprechtsofen). Finally, one cave (Gamsställe) yielded a Quaternary age and thus confirms the assumption that the corresponding sediment was washed in during a glacial highstand.

The obtained valley deepening rates are in good agreement with results from the older parts of Siebenhangste and with the conclusion of Frisch et al. (2008). Deepening rates that can be used are between 0.12 to 0.21 km/Ma, and rates increase from Miocene towards Quaternary.

ACKNOWLEDGEMENTS

First thanks go to Herta Effenberger (University of Vienna) who let us use space for a laboratory, as well as the whole Geozentrum at Vienna University for the help and welcome to our project. The PrimeLab of Purdue University (Indiana, USA) is thanked for sample processing and measuring the isotopes. The Austrian Science Fund FWF is thanked for funding the project (P 19362-N10). Contributions from Diana Sahy (Fieldwork and partial treatment of the samples) and Helene Pfalz-Schwingenschlögl (figure drawings) were instrumental in obtaining the results and the present article. Philippe Audra and Jo de Waele are thanked for thoughtful reviews.

Authorship statement: PH and MF designed and directed the study. PH calculated the ages, LP and PP were involved in fieldwork and field interpretation. PH and LP wrote the paper with substantial input form PP.

REFERENCES


Speleothems in quartz-sandstone caves of Ponta Grossa municipality, Campos Gerais region, Paraná state, southern Brazil

Henrique S. Pontes 1,2*, Luiz A. Fernandes 3, Mário S. de Melo 1, Gilson B. Guimarães 1,2, and Laís L. Massuqueto 3,2

1Geoscience Department, Ponta Grossa State University (UEPG), Av. General Carlos Cavalcanti, 4748, Ponta Grossa, Brazil
2University Group of Speleological Research (GUPE), St. São Romulo, 375, Ponta Grossa, Brazil
3Federal University of Paraná (UFPR), Av. Cel. Francisco H. dos Santos, 100, Curitiba, Brazil

Abstract: The present communication characterizes and discusses the genesis of cave speleothems developed in Furnas Formation (Silurian-Devonian) and Vila Velha sub-unit (Campo Mourão Formation, Upper Carboniferous) quartz-sandstones, in southern Brazil. The research, which involved stratigraphic and petrographic studies, was conducted to identify the faciological context of occurrence of these features, and to determine, through different analytical methods, their mineralogical composition and porosity, and also the action of microbial markers on the genesis of these secondary mineral deposits. The speleothems were classified into six types based on form, composition and genesis: (1) arborescent (coral), (2) mushroom-shaped, (3) stalactitic, (4) stalagmitic, (5) columnar with horizontal/ascending growth, and (6) fibrous. In response to substrate permoporosity, speleothems develop on all sandstone facies of the study area, mainly in sites controlled by bedding, stratification and fracturing planes. They are less frequent, however, in conglomerate and silty-clayey layers of the Furnas Formation, being composed mainly of silica (opal-A and cryptocrystalline silica) and kaolinite, with lesser contribution of gypsum and amorphous iron oxides associated with goethite and hematite. Detritic minerals such as quartz and muscovite also take part in the composition of these features. Plant and invertebrate fragments were identified, including biosignatures and microorganisms capable of favoring precipitation of silica (possibly cyanobacteria and diatom) and iron oxide (possibly Gallionella ferruginea). Such biosignatures and extracellular polymeric substances (biofilms) associated with speleothems are evidence of organomineralization, also known as biologically induced mineralization. The results, which indicate biological action on the genesis of speleothems, raise further discussion on the influence of microorganisms on the dissolution and precipitation of silica and kaolinite in the region.

Keywords: microbialites, non-carbonate rock caves, microorganisms, Furnas Formation, Vila Velha Sandstone, speleothems

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INTRODUCTION

Chemical weathering is a determining factor to the development of relief forms in karst systems. Through chemical processes such as hydration, dissolution, hydrolysis and oxidation, ions and molecules are removed from the surfaces of mineral components, allowing thus the formation of secondary porosity by karstification, with partial or total removal of constituents (Dubois et al., 2014). In both cases, removal and transportation of ions and molecules creates cavities and speleothems, the latter corresponding to secondary chemical deposits.

Such geological features usually form in underground environments, but they are also found in superficial portions of karst systems, such as cavities on rock walls. However, there are reports of speleothems in areas subject to chemical weathering and mineral precipitation in caves or surface walls where karst relief is absent, as are granite (e.g., Vidal Romani & Rodriguez, 2007) and basalt (e.g., Miller et al., 2014; Gonzalez-Pimentel et al., 2018) outcropping areas.

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Being unexclusive of karst systems, speleothems are not restricted to carbonate rocks. The development of these features is perfectly possible in rocks of low solubility, as are those of siliciclastic composition like quartz-sandstones and quartzites. In fact, several non-carbonate karst systems around the world are located in quartzites and quartz-sandstones with features, shapes, spatial organization and functioning characteristic of karst areas, as reported by Wray & Sauro (2017).

Several karst occurrences in Brazil are found in siliciclastic rocks whose dissolution features include speleothems. Main examples are quartzites of the Itacolomi Group in the State of Minas Gerais (Renó et al., 2009), quartz-sandstones of the Chapada dos Guimarães plateau in the State of Mato Grosso, and Serra de Itaqui, State of São Paulo (Hardt et al., 2009), sandstone caves in the State of Tocantins (Morais & Souza, 2009), and quartzite caves along the Serra do Itibipoca range, State of Minas Gerais (Corrêa Neto & Baptista Filho, 1997; Willens et al., 2008). One of the most notable examples of karst in siliceous rocks is located in South America, in the Tepuis of southeastern Venezuela, where gigantic cave systems include siliceous speleothems of large dimensions, diversity and rarity (White et al., 1966; Urbani, 1990; Galan & Lagarde, 1988; Aubrecht et al., 2012; Letenski et al., 2011) and quartz-sandstones of the Chapada dos Guimarães plateau in the State of Mato Grosso, and Serra de Itaqui, State of São Paulo (Hardt et al., 2009), sandstone caves in the State of Tocantins (Morais & Souza, 2009), and quartzite caves along the Serra do Itibipoca range, State of Minas Gerais (Corrêa Neto & Baptista Filho, 1997; Willens et al., 2008). One of the most notable examples of karst in siliceous rocks is located in South America, in the Tepuis of southeastern Venezuela, where gigantic cave systems include siliceous speleothems of large dimensions, diversity and rarity (White et al., 1966; Urbani, 1990; Galan & Lagarde, 1988; Aubrecht et al., 2012; Letenski et al., 2011; Sauro, 2014; Sauro et al., 2018, 2019; Auier & Sauro, 2019).

In non-carbonate cemented quartz-sandstones of Furnas Formation and Vila Velha Sub-unit terrains in southern Brazil, karstification involves dissolution of quartz and other mineral components. These rocks host hundreds of caves, dolines, underground drainage systems with sinks and springs, among other geological features typical of karst relief i.e., arenized/phantomized rocks, ceiling and wall channels, dissolution ducts, domes, and alveoli.

Speleothems are also present in these units, mainly inside caves. Several authors report such deposits in Furnas Formation, among whom Spinardi and Lopes (1990), Budny (2004), Melo and Giannini (2007), Pontes et al. (2008), Melo (2010), Pontes (2010, 2014), Massuqueto (2010), Melo et al. (2011, 2015), Hardt (2011), and Flügel Filho (2012). Few mentions are made of the such deposits in Vila Velha sandstones, though. Melo (2006), Letenski et al. (2009) and Letenski et al. (2011) mention micropaleoethems that occur at surface level. Pontes et al. (2012) report a set of speleothems up to 15 cm long in an underground cavity in the same rocks.

Quartz-sandstone speleothems exhibit, in most cases, characteristics that are different from those developed in carbonate rocks. In abundance and size, they are usually smaller compared to their carbonate counterparts. However, secondary chemical deposits in quartz-sandstones are among the most intriguing geological features, mainly due to their composition and genesis.

Flügel Filho et al. (2012) and Flügel Filho (2012) present a preliminary morphological classification for speleothems developed on Furnas Formation rocks. Melo et al. (2015) point out a siliceous composition revealed by Energy-dispersive X-ray Spectrometry (EDS). The study carried out by Melo et al. (2015) is the only one to present the chemical analysis of these secondary mineral deposits in rocks of the Furnas Formation, which does not correspond, however, to the speleothems presented here. Studies on speleothems of the Vila Velha Sub-unit have not yet been performed.

Information gaps regarding secondary chemical deposits in Furnas Formation and Vila Velha Sub-unit quartz-sandstones would justify detailed studies involving microscopy, chemical and petrographic analysis, and stratigraphic surveying to identify possible correlations between the development of such features and the faciological and diagenetic characteristics of the sandstones. The present study aims to characterize cave speleothems developed in both geological units, discussing the genesis of these features and the possible influence of microorganisms on quartz and kaolinitic cement dissolution and precipitation of secondary minerals as well.

**STUDY AREA**

The research was conducted on data from underground cavities found in Furnas Formation (Silurian-Devonian) and Vila Velha Sandstone rocks, the latter a sub-unit of the Campo Mourão Formation (Carboniferous-Permian) in the eastern border of the Paraná Basin, in Ponta Grossa municipality, State of Paraná, southern Brazil (Fig. 1).

One hundred and ten caves are found in the study area, 106 of which in rocks of the Furnas Formation, and four in Vila Velha sandstones, along with hundreds of dolines and sinks, springs, underground rivers, erosive features (speleogens) and arenized/phantomized rocks, all of them typical of karst relief. In non-carbonate quartz-sandstones, karstification process involves dissolution of kaolinitic cement and quartz grains, as highlighted by Pontes (2010) and Melo et al. (2011, 2015).

The regional climate is identified as Subtropical Cfb in Köppen’s classification, corresponding to mild temperate conditions with average temperature below 18°C in the coldest month and below 22°C in the hottest month. Summers are cool, with no definite dry season. Rainfall is well distributed, with average annual rainfall between 1,400 and 1,800 mm, and average relative humidity between 75 and 80% according to Cruz (2007). It is worth mentioning the presence, in the eastern borders of the study area, of the topographic step represented by Devonian Escarpment delimiting the first two plateaus of Paraná and whose altitude differences, of up to 300 m at some points, allow for a localized increase in precipitation and air humidity.

**GEOLOGICAL ASPECTS**

Terrains including mostly quartz-sandstones of the Furnas Formation (Paraná Group) and the Vila Velha Sub-unit of the Campo Mourão Formation (Itararé
Speleothems in quartz-sandstones caves, Brazil
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Fig. 1. Location of the study area. Cartographic conventions: (1) caves; (2) dolines; (3) municipal limits of Ponta Grossa; (4) Devonian Scarpment; (5) outcrop area of the Itararé Group including the Vila Velha Sandstone sub-unit; (6) outcropping area of the Furnas Formation. Numbers indicate caves from which samples were collected: (1) Fuma do Buraco do Padre; (2) Caverna da Chimine; (3) Gruta da Inspirada; (4) Caverna do Zé; (5) Sumidouro do Córrego das Fendas; (6) Fenda Sem Fim; (7) Gruta Andorinhas da Pedra Suspensa.

Group) extend for approximately 85 and 15% of the total study area, respectively.

The Furnas Formation sediments are Silurian-Devonian in age, dating between 421 and 395 Ma (Borghi, 1993; Assine, 1999; Milani et al., 2007). They consist mainly of medium to coarse quartz-sandstones cemented by kaolinite and illite (Assine, 1996; Melo & Giannini, 2007 and Milani et al., 2007). The sandstones bear plane-parallel and cross-stratifications and, commonly, decimetric intercalations of silt and clay. At the base of the formation, conglomerate layers exceed one meter thick.

According to Assine (1996), the sandstones occur in tabular, lenticular and cuneiform strata with thickness between 0.5 to 5 m and marked cross-stratification. The same author proposes a subdivision of Furnas Formation in three, Lower, Middle and Upper units. Another stratigraphic classification is proposed by Lobato & Borghi (2005), according to which the formation is compartmentalized into four units, the Interval II of Assine (1996) being individualized as two segments, A and B. According to Assine (1996) the Furnas Formation is about 250 m thickness.

Bergamaschi (1992), Assine et al. (1994) and Assine (1996) suggest deposition of the Furnas Formation under deltaic and platformal conditions. However, much remains to be discussed on the deposition of these units, especially regarding the lower conglomeratic section, which is totally devoid of fossils (Milani et al., 2007). Some authors argue that the deposition of the Furnas Formation occurred in anastomosed river systems (Schneider et al., 1974; Zalán et al., 1987) or in a large braided river system (Pavan, 2015; Araújo, 2016).

The Vila Velha Sandstone lies concordantly over diamicites, glacial rhythms and argilites of the Campo Mourão Formation. The sub-unit consists essentially of fine to coarse reddish sand grains welded by a ferruginous cement, with varying degrees of sorting, and the presence of pebbles at the base. Maack (1946) identifies quartz, partially kaolinized feldspar, muscovite, chlorite and garnet among the mineral constituents of the formation. Melo and Coimbra (1996) and Melo et al. (2007) point out, however, that these minerals occur in layers below the sandstones that known for the typical relief of
the Vila Velha region. The latter unit would consist only of quartz-sandstones with a reduced amount of polycrystalline grains (quartzite) and fragments of sandy mudstones forming a pseudomatrix that resulted from deformation and crushing. Schneider et al. (1974) compartmentalize the Itararé Group in the Campo do Tenente, Mafra and Rio do Sul formations. França and Potter (1988) propose another division in which the unit includes the Lagoa Azul, Campo Mourão and Taciba formations, this being the classification adopted for the present study. Maack (1946), Melo and Coimbra (1996), França et al. (1996), and Melo et al. (2007) report incipient plane-parallel stratification in these sandstones, which are normally massive with low-angle cross-stratification, wavy marks and clayey intraclasts. According to França et al. (1996), the sandstones exhibit the well-known ruiniform relief of the Vila Velha region, consisting in layers more than 30 m thick and hundreds to more than a thousand meters wide. For Melo et al. (2007), the thickness of Vila Velha Sandstone layers is of the order of 50 m, whereas for Maack (1946) it corresponds to 65 m. Vesely (2011) includes an additional 30-m-thick arenitic interval, referred to as the Basal Sandstone. According to Milani et al. (1994), the rocks comprised in the Itararé Group were deposited in fluvioglacial, marine-glacial and marine environments. França et al. (1996) consider the Vila Velha sandstones as deposited in the form of subaqueous lobes under dense gravitational flows initiated at basal parts glaciers. The occurrence of low-angle cross-stratification and vertical and horizontal perforations indicates shallow marine sedimentation influenced by tides (Canuto et al., 1997). Vesely (2011) identifies grooves and glacial ridges in the stratigraphic succession of the Vila Velha Sandstone, suggesting glacier displacement over unconsolidated sediments, which is characteristic of proglacial environments. An Upper Carboniferous age is attributed to The Vila Velha Sandstone based on the fossil content of shales that occur interspersed with sediments of other Campo Mourão Formation sub-unit, the Lapa Sandstone, and correlations with rocks of the Lagoa Azul Formation (Milani et al., 1994). The relief in exposed areas of Furnas and Vila Velha rocks is markedly controlled by tectonic structures associated with canyons and crevices along two main directions. According to Załán et al. (1990), these structures resulted from the uplift of the Ponta Grossa Arch (NW-SE) and reactivation of Proterozoic basement terrains (NE-SW) by crustal arching during the Mesozoic. Such tectonic structures facilitate weathering and the erosive action of water at surface and underground environments, which makes such discontinuities preferred zones for the development of underground ducts and galleries.

METHODS

Surveying consisted in fieldwork to collect information on lithotypes, preparation of geological sections, and identification of speleothems in underground cavities and rocky walls. Occurrences of these secondary chemical deposits were identified against an inventory of geodiversity compiled for cavities of the study area by Pontes et al. (2018a, b). Laboratory work included stratigraphic interpretation, petrographic interpretation, faciological characterization, and chemical analysis. A tentative correlation between the speleothems and stratigraphic and lithological characteristics of the sandstones is drawn based on geological sections of occurrence sites. Six attributes were considered: (1) mineralogical composition and textural parameters (grain size and sorting), (2) porosity, (3) sedimentary structures, (4) tectonic structures, (5) weathering features (arenization/phantomization), and (6) pattern of occurrence.

Different analytical methods were applied to characterize the speleothems (Table 1). In order to determine their internal structures and to identify their distinct components, especially those of biological origin, Transmitted Light Microscopy (Leica DMLP, with polarizers and digital image capture) was initially applied, at Federal University of Paraná facilities, followed by Scanning Electron Microscopy (SEM). Total porosity values were interpreted from Computed Tomography (micro-CT) images obtained with Skyscan 1172 equipment at 88 kV of voltage and 112 µA of current intensity at the Laboratory of Minerals and Rocks (LAMIR) of the same university. The Skyscan CTVox software version 1.5 performs an automatic count of the black pixels (interpreted as pores) of the micro-CT binary images, providing a mathematical estimate of the total porosity of the material.

In order to identify a possible contribution of microorganisms to the genesis of the secondary deposits, SEM was applied, with Tescan VEGA3 equipment at the Electron Microscopy Center (CME) of the Federal University of Paraná (UFPR) and Shimadzu SSX-550 equipment at the Multi-User Laboratory Complex (CLabmu) of the Ponta Grossa State University (UEPG). In both cases, the voltage used was 15 kV. At that research stage, physical (form) and chemical (composition) similarities between the observed speleothems and those of the largest cavities in Precambrian sandstones of the Venezuelan Tepuis were drawn. As Aubrecht et al. (2008) and Lundberg et al. (2010) point out, the Tepuis speleothems, which correspond to microbialites or biospeleothems, were formed under the influence of microorganisms.

For control and identification of the chemical elements present in the samples that were subject to SEM, Energy-dispersive Spectrometry (EDS) was applied with tools coupled to the microscopes. The chemical composition of the speleothems, in turn, was determined by Powder X-ray Diffractometry (XRD) with a D8 Advance DaVinci Bruker diffractometer (CuKα, 40 kV, 40 mA, 3 to 70 degrees 2θ) at the X-ray Diffraction Laboratory of the Geosciences Institute, University of São Paulo (USP).

Four samples of speleothems developed in Vila Velha sandstones were analyzed, all from a cave known...
Table 1. Samples, performed analyses, and collection sites. Samples highlighted in gray are from Formação Furnas whereas the orange ones from Vila Velha Sandstone.

<table>
<thead>
<tr>
<th>Identification</th>
<th>Material Description</th>
<th>Analysis</th>
<th>Collection Site</th>
<th>Color</th>
</tr>
</thead>
<tbody>
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<td>1 BP-01</td>
<td>mushroom-shaped speleothem</td>
<td>XRD</td>
<td>Cave, entrance area</td>
<td>white/gray</td>
</tr>
<tr>
<td>2 BP-02</td>
<td>mushroom-shaped speleothem</td>
<td>SEM/EDS</td>
<td>Cave, entrance area</td>
<td>white/gray</td>
</tr>
<tr>
<td>3 CC-01</td>
<td>arborescent speleothem</td>
<td>Petrographic Microscopy</td>
<td>Cave, twilight area</td>
<td>gray/brown</td>
</tr>
<tr>
<td>4 CC-02</td>
<td>stalactitic speleothem</td>
<td>Petrographic Microscopy</td>
<td>Cave, twilight area</td>
<td>gray/brown</td>
</tr>
<tr>
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<td>SEM/EDS</td>
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</tr>
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<td>red/yellow</td>
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<td>stalagmitic speleothem</td>
<td>SEM/EDS</td>
<td>Cave, dark area</td>
<td>red/yellow</td>
</tr>
<tr>
<td>11 GI-01</td>
<td>arborescent speleothem</td>
<td>SEM/EDS/XRD</td>
<td>Cave, dark area</td>
<td>brown/white</td>
</tr>
<tr>
<td>12 SCF-01</td>
<td>columnar speleothem with horizontal/ascending growth</td>
<td>SEM/EDS/XRD</td>
<td>Cave, dark area</td>
<td>brown/white</td>
</tr>
<tr>
<td>13 SCF-02</td>
<td>columnar speleothem with horizontal/ascending growth</td>
<td>Petrographic Microscopy</td>
<td>Cave, dark area</td>
<td>brown/white</td>
</tr>
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<td>14 SCF-03-A</td>
<td>columnar speleothem with horizontal/ascending growth</td>
<td>Micro-CT</td>
<td>Cave, dark area</td>
<td>brown/white</td>
</tr>
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<td>15 SCF-03-B</td>
<td>columnar speleothem with horizontal/ascending growth</td>
<td>Petrographic Microscopy</td>
<td>Cave, dark area</td>
<td>brown/white</td>
</tr>
<tr>
<td>16 SCF-04</td>
<td>mushroom-shaped speleothem</td>
<td>SEM/EDS</td>
<td>Cave, dark area</td>
<td>white/gray</td>
</tr>
<tr>
<td>17 SCF-05</td>
<td>rimstone (micro gours)</td>
<td>SEM/EDS</td>
<td>Cave, dark area</td>
<td>brown/red</td>
</tr>
<tr>
<td>18 FSF</td>
<td>fibrous speleothem</td>
<td>XRD</td>
<td>Cave, dark area</td>
<td>yellow/white</td>
</tr>
<tr>
<td>1 GAPS-01-A</td>
<td>arborescent speleothem</td>
<td>Petrographic Microscopy/ Micro-CT/XRD</td>
<td>Cave, entrance area</td>
<td>gray/brown</td>
</tr>
<tr>
<td>2 GAPS-01-B</td>
<td>arborescent speleothem</td>
<td>SEM/EDS</td>
<td>Cave, entrance area</td>
<td>gray/brown</td>
</tr>
<tr>
<td>3 GAPS-02-A</td>
<td>mushroom-shaped speleothem</td>
<td>XRD</td>
<td>Cave, entrance area</td>
<td>gray/white</td>
</tr>
<tr>
<td>4 GAPS-02-B</td>
<td>mushroom-shaped speleothem</td>
<td>SEM/EDS</td>
<td>Cave, entrance area</td>
<td>gray/white</td>
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as Gruta Andorinhas da Pedra Suspensa, located within a protection area in the State of Paraná, the Vila Velha State Park (Fig. 1). Specific studies were carried out on 18 samples of secondary chemical deposits from Furnas sandstones, among which rimstones (microterraces or micro-gours) that are not characterized as speleothems in the study area. The collection points at Furnas Formation outcrops are all located within the Campos Gerais National Park, in the following caves: Furna do Buraco do Padre, Caverna da Chaminé, Gruta da Inspirada, Caverna do Zé, Sumidouro do Córrego das Fendas, and Fenda Sem Fim (Fig. 1).

**RESULTS**

**Classification**

Speleothems occur in nearly all underground cavities found in the study area, along with alveoli, recesses and ducts in surface cavities. Six varieties of speleothems have been defined upon revision of the classification presented by Flügel Filho et al. (2012) and Flügel Filho (2012): a) arborescent (coral), b) mushroom-shaped, c) stalactitic, d) stalagmitic, e) columnar with horizontal/ascending growth, and f) fibrous (Fig. 2 and Fig. 3). Although small in size compared to those developed on carbonate rocks, and ranging between a few millimeters and 15 centimeters in length, the speleothems of the study area are abundant and easily found. Of reduced dimensions, the rare fibrous speleothems present are exceptions.
Arborescent speleothems are the most commonly found in the study area. They occur as main structures that branch into dendritic segments, usually with irregular or rugged surfaces. The mushroom-shaped speleothems resemble pileus (hats) of some varieties of fungi, usually in convex shapes. They occur alone or in groups, also with rugged or smooth surfaces.

Stalactitic speleothems usually project downward from cave ceilings as columnar forms with rugged or smooth surfaces. Stalagmitic ones rise from the floor in rough or smooth convex forms whose origin is usually associated with dripping water, occurring predominantly in red, but also in orange and black shades in some portions. Columnar speleothems of horizontal/ascending growth form on cave walls and floors, usually in rugged columnar shapes. The fibrous one consists of filamentous crystal clusters with irregular surfaces, in yellowish-white colors. Except for the stalagmitic and filamentous ones, the speleothems can assume various shades of white, gray and brown. However, their color relates to the presence of coloring mineral components such as iron oxide, and not to morphological characteristics.

**Composition**

Similarly as in speleothems of the Vila Velha sandstones (Fig. 4b), silicon and aluminum form the arborescent, mushroom-shaped, stalactic and columnar with horizontal/ascending grow speleothems found in Furnas Formation quartz-sandstones (Fig. 4a). In the present study, X-ray diffraction indicates the presence of opal-A, cryptocrystalline silica and cryptocrystalline kaolinite (Fig. 4c-f) as mineral components. The presence of Opal-A was diagnosed by comparison with siliceous speleothems of other regions of the globe (e.g., Finlayson & Webb, 1985; Wray, 1999; Aubrecht et al., 2008). The quartz peaks present in the graphs, in addition to being the result of the presence of detritic quartz, represent cryptocrystalline silica, possibly chalcedony.

Muscovite is also present, in detritic fragments weathered and transported from silt and clay layers by water and imprisoned in biofilms.

The stalagmitic speleothems identified in the caves known as Caverna do Zé and Caverna das Andorinhas (Furnas Formation) are composed entirely of iron oxides, specifically amorphous varieties associated with goethite and hematite (Fig. 5a, b). Fibrous speleothems occur only as a small set of features, in a cave known as Fenda Sem Fim, developed in rocks of the Furnas Formation (Fig. 5c). An unprecedented fact is the presence of speleothems formed solely by gypsum in rocks of the Furnas Formation. Being this a unique and small occurrence, only material for determination of chemical composition by X-ray diffraction could be collected (Fig. 5d).

**Biological records**

SEM images revealed the presence of several biosignature elements including molds, fragments and even complete structures of past microorganisms in speleothems developed on Furnas Formation and Vila Velha Sub-unit rocks. Such biological markers are present in massive portions of speleothems, composed of silica, being also considered the most important records of microbial action in the study area (Fig. 6a, b). In some speleothems, clusters possibly formed by cyanobacteria appear as filamentous biofilms segmented by globules (Fig. 6c, d).

Several speleothem samples present fully preserved microorganisms, possibly corresponding to fossil structures (Fig. 6e). Also, other biological organisms
Fig. 4. Energy-dispersive spectrometry analysis of cave speleothems. Silicon and aluminum: a) Gruta da Inspirada, Furnas Formation; b) Gruta Andorinhas da Pedra Suspensa, Vila Velha Sandstone; c) X-ray diffraction analysis indicating opal-A (Finlayson & Webb, 1985) and kaolinite as mineral components of speleothems of the Sumidouro do Córrego das Fendas, Furnas Formation; d) Gruta Andorinhas da Pedra Suspensa, Vila Velha Sandstone; e-f) Speleothemes composed of cryptocrystalline silica.

Fig. 5. a) Energy-dispersive Spectrometry analyses indicating iron oxide in the composition of stalagmitic speleothems found at Caverna do Zé, a cave in Furnas Formation rocks; b) X-ray diffraction defining the composition of the same secondary mineral deposit, iron amorphous oxide associated with hematite and goethite; c) small set of fibrous speleothems in the Fenda Sem Fim cave system, Furnas Formation; d) X-ray diffraction results indicating gypsum in the composition of said deposit.
were trapped during chemical precipitation, especially in the initial stages of formation. Remnants of biological materials such as plant fragments, invertebrates (whole shells and molds), diatoms, amoeba and many types of fungal and plant spores were also identified (Fig. 6f-i; Fig. 7a-h).

**Fig. 6.** Secondary SEM images showing biological components in Furnas and Vila Velha Sandstone speleothems: a) bacteria coci (or archea); b) different bacteria grouped as globular filaments surrounded by secondary precipitation of silica, and possibly Bacillus sp. on the right of the image; c) biosignatures in secondary precipitation of silica; d) various microscopic organisms forming a biofilm; e) completely silicified mite; f) frustule of possible diatom Aulacoseira sp.; g) plant fragment similar to a microleaf; h) unidentified diatom; i) Testate Amoebae possibly Euglypha sp.

**Internal structures**

Total porosity values obtained by visual quantification under petrographic microscopy vary according to the type of speleothem, even at hand sample scale when distinct internal sectors of the feature are considered. Mushroom-shaped speleothems are the most porous ones compared to other varieties. Conversely, speleothems bearing well-defined internal microlaminations, as do arborescent, stalactitic and columnar ones tend to present low total porosity values.

Under micro-CT analysis, a columnar speleothem with horizontal growth from Sumidouro do Córrego das Fendas cave (Furnas Formation) showed a total porosity of approximately 7%, while an arborescent deposit in the cave known as Gruta Andorinhas da Pedra Suspensa (Vila Velha Sandstone) presented a value of 3.7% for the same property (Fig. 8). However, total porosity tends to be higher in the outermost portions of speleothems, reaching 27.4% in a Furnas Formation specimen, and 7% in another one developed on Vila Velha sub-unit. The same aspect was revealed by Transmitted Light Microscopy (Fig. 9a, b).

Three, inner, intermediate and outer microfacies could be identified in arborescent, stalactitic and columnar with horizontal/ascending growth speleothems by applying Transmitted Light Optical Microscopy to thin sections perpendicular to older, inner microlaminations, and thin sections perpendicular to younger, outer microlaminations of speleothems (Fig. 10a).

In the inner microfacies, well-defined overlapping microlaminations indicate the growth direction of the speleothem (Fig. 10b). Colors vary between brown and white, the laminae being evenly spaced from one another. Quartz grains, and muscovite lying concordantly to lamination are the main constituents. As the deposit took shape, mineral grains adhered to surface irregularities and biofilms.

Lamination in the intermediate microfacies is incipient, ranging from dark brown to white. Laminae are thicker compared to those of the inner microfacies (Fig. 10c). Pores and biological components, mostly represented by plant fragments, are more frequent.

Microlaminations are not found in the outer microfacies, that presents itself with a disorganized
aspect. Dark brown shades predominate, with sparse light portions (Fig. 10d). Porosity and the amount of biological components, especially plant fragments, are higher compared to the other microfacies (Fig. 10e-h).

In some cases, light portions of the outer microfacies present wavy or fringed laminae similar to those described in stromatolites of carbonate sedimentary rocks by Iespa et al. (2012).

Petrographic analysis of thin sections of a small portion measuring 1.07 cm in length of an arborescent speleothem revealed the presence of 78 laminae, 39 dark and 39 light ones (Fig. 10i). Lamination thickness ranges from 20 μm to 1.93 mm. The thinnest laminae are found in the inner microfacies, while the thicker ones are concentrated in the outer microfacies. The alternation of colors between laminated pairs is given by differences in speleothem accretion phases, possibly related to periods of higher or lower humidity.

Stalagmitic speleothems present two distinct interspersed microfacies (Fig. 11a, b), one with a tenuous microlamination from red to yellow shades (Fig. 11c), and another one, predominant in this type of speleothem, composed of well-defined red to black laminae below reddish to orange columnar microstructures (Fig. 11d). These columnar microstructures correspond to portions of high bacteria concentration, possibly Gallionella ferruginea, as revealed in SEM images.

Mushroom-shaped forms do not have a defined internal structure pattern, possibly because they correspond to initial stages of speleothem development, are in full genetic activity. Due to low physical resistance, thin sections of these materials could not be mounted. However, in scanning images they show to be disorganized deposits devoid of microlaminations (Fig. 11e).

Patterns of occurrence

Geological sections and faciological characterization of the rocks that form the substrate to speleothems show that these features develop in all sandstone lithofacies regardless of granulation, sorting or degree of cementation. Less often, and to a lesser extent, these
deposits occur in silty-clayey layers and conglomerate strata of the Furnas Formation.

Bedding, stratification and fracturing planes exert evident control over the development of speleothems. In some cases, weathering features are associated with neoformed mineral deposits. This does not constitute a rule, however, since speleothems are present in both friable and diagenetically silicified portions of the sandstones.

As already mentioned, speleothems develop in underground and surface cavities, from aphotic to dim to fully illuminated environments (Table 1). Conversely, humidity and temperature exert direct influence on the formation of these features. Also, it is important to highlight the fundamental role played by aerosols in the formation of speleothems in underground cavities, as already pointed out by Klimchouck et al. (1997), Maltsev (1997), and Dredge et al. (2013).

**Deposits like rimstones**

Features known as rimstone are found in outcropping areas of Furnas Formation and Vila Velha sandstones. Some descriptions of the study area report such features as speleothems (e.g., Massuqueto, 2010; Pontes, 2010; Flügel Filho et al., 2012). However, petrographic analysis and SEM reveal that their characteristics are not of secondary chemical, but clastic deposits.

Deposits like rimstones are formed by displacement of soils and organic matter from the surface of the terrain into caves by water that infiltrate sedimentary and tectonic discontinuities. Water and sediments flow through the walls of caves forming cascade-like accumulations and rigid crusts after circulation ceases.

The clastic deposits thus formed vary in grain size, but with a predominance of silt and clay. The relative plasticity determined of silty and clayey fractions allows deposits to manifest in typical shapes due to the constant flow of water and sediments over previously existing layers, in a process described by Vidal Romani et al. (2014) in granite caves.

These features developed on vertical walls, where water flow is more intense, exhibit rugged surfaces and varied concave, convex, fringed shapes forming...
microterraces. Over ramps, where water flow is less intense, small dams and pools (micro-gours) commonly form due to the slow and irregular displacement of water on the sediments.

Even though the studied specimens do not show speleothem characteristics, the possibility of mineral precipitation in such deposits is not excluded, their outer portions being worth of detail studies, especially where rigid crusts are present. Also, such features are highly capable of capturing components of biological origin, thus potentially serving as paleoenvironmental and paleoecological markers.

**DISCUSSION**

**Genetic aspects**

Melo et al. (2015) point out that in rocks of the Furnas Formation the arborescent, mushroom-shaped, stalactitic and columnar with horizontal/ascending grow speleothems varieties are composed of amorphous to cryptocrystalline silica and cryptocrystalline kaolinite. Several studies point to continuous evaporation cycles as triggers of silica precipitation, indicating that speleothems only develop in areas with free circulation of air under unsaturated atmosphere (e.g., Webb & Finlayson, 1984; Hill & Forti, 1997; Wray, 1999). However, clusters of globular microbial filaments (possibly cyanobacteria) were found in samples, forming extracellular polymeric biofilms (Fig. 12a). Such biosignatures show features that result from microbiological action, which allows such secondary chemical deposits to be classified as microbialites or biospeleothems. The genesis of speleothems is, therefore, induced or influenced by microorganisms.

Several studies discuss the influence of microbial metabolic activity on the precipitation of silica (e.g., Cañaveras et al., 2001; Forti, 2001; Aubrecht et al., 2008; Loves & Warren, 2009; Daza Brunet & Bustillo Revuelta, 2014; Lundberg et al., 2018). Similarly, some microorganisms are also capable of modifying chemical conditions of rocks at the microenvironment scale (millimeter by centimeter), thus possibilitating local dissolution of silica (Bennet, 1991; Ehrlich, 1996; Brehm et al., 2005; Vidal Romani & Rodriguez, 2007; Miot et al., 2014).

According to Baskar et al. (2007); Sallstedt et al. (2014); Zepeda Mendoza et al. (2016), biofilms play an important role in the genesis of some speleothems. The presence of biofilms associated with cave speleothems in the study area such as in Furna do Buraco do Padre (Furnas Formation) and Gruta Cambiju I (Vila Velha Sandstone) implies biological action in the development of these geological features (Fig. 12b). Thus, SEM proved to be an efficient method to detect biosignatures, as pointed out by to Miot et al. (2014).

Not only are polymeric extracellular substances able to adhere to particles that touch the biofilm such as mineral grains and biological organisms or fragments, but they can also promote precipitation of secondary minerals such as silica, kaolinite and iron oxides. Figure 13 outlines the formation of arborescent, stalactitic, columnar with horizontal/ascending growth, mushroom-shaped speleothems, showing that the process starts with water percolating the rock and the removal of ions and molecules from the surface of mineral components by various chemical processes. Subsequently, the solution reaches the biofilm, where microbial metabolic processes promote the precipitation of chemical elements to form speleothems. Overlapping microlaminae are accreted to the speleothem as the cycle repeats, a process not observed only in mushroom-shaped speleothems, which correspond to deposits in early development stages.

In addition to microbial action, the development of some speleothems may be influenced by the presence of spider webs, especially in arborescent types. Field observation allowed to identify the action of different spiders species on speleothems. Aubrecht et al. (2008) and Lundberg et al. (2010) report mineral precipitation
along web strands, forming what those authors refer to as cobweb stalactites. Silica precipitation along spider web strands was identified in Furnas and Vila Velha sandstone speleothems, evidencing that these filaments can serve as preferential sites for the mineral incrustation (Fig. 14a, b). Elongated structures up to 5-10 μm in diameter interpreted as originally corresponding to web strands were identified on thin petrographic sections. According to Pontes et al. (2018a), at least five families of these invertebrates distributed over five different morphospecies produce webs in speleothems of the study area. Stalagmitic speleothems result from dissolution of ferruginous incrustations along fractures and faults. Water rich in solubilized iron infiltrates the cave ceiling, accumulating such components over
the ground usually by dripping. These speleothems can be classified as microbialites given the presence of numerous clusters of bacteria, possibly *Gallionella ferruginea* (Fig. 14c, d). These microorganisms produce polymeric extracellular substances in the form of twisted ribbon-like stems on which iron oxide precipitates, as reported by Søgaard et al. (2001), Hallberg & Ferris (2004), Zhilei et al. (2010), and Chan et al. (2011).

Owing to their rarity, fibrous speleothems constitute secondary mineral deposits of high scientific value in the region of Campos Gerais do Paraná. Based on comparisons of color and habit, Pontes et al. (2017) report similar speleothems only in the cavity known as Abrigo Casa de Pedra da Granja, formed in rocks of the Itararé Group in Tibagi municipality, 90 km from the study area.

In a gypsum speleothem found in the cave known as Fenda Sem Fim (Furnas Formation), the source of sulfate may correspond to the guano of bats and biscutate swifts (*Streptoprocne biscutata*), the latter a typical species in the region. Oxidation caused by fungi and bacteria is able to promote precipitation of sulfate from decomposing organic matter, as pointed out by Vidal Romaní et al. (2010) and Aubrecht et al. (2012). The chemical alteration of feldspars present in Furnas Formation sandstones (Borghi, 1993) may represent the source of calcium. Sauro et al. (2014) point to the possibility that microbially reduced atmospheric sulfate from forests or peatlands be carried as aerosols to the interior of caves by air streams. However, the reduced occurrence in one single cave leaves the origin of such secondary mineral deposits open for discussion. Specific microclimatic conditions of temperature, humidity and luminosity combined with the availability of chemical elements (Ca and S) are deemed fundamental factors to the formation of this variety of speleothems.

The speleothems alternating laminae are of potential use as annual or seasonal climatic markers, as pointed out by Sallstedt et al. (2014), Riechelmann et al. (2016), and Zepeda Mendoza et al. (2016). Regarding differences in porosity (low porosity in the innermost portions), they possibly result from the time interval elapsed during speleothem accretion. Older, internal sectors tend to be less porous and better microlaminated than external ones. Decomposition of organic matter, mineral substitution and compaction are factors related to low porosity. In some cases, porosity also results from microbial action, involving mineral precipitation inside speleothems due to current biological colonization by diatoms, bacteria and other microorganisms able to promote precipitation of silica. The alternation of colors among laminae may also be related to differences in mineralogical composition. In siliceous speleothems of Australian caves, Wray (1999) noticed an alternation of light laminae composed of chalcedony and dark laminae composed of chalcedony and opal-A/chalcedony.

Studying speleothems developed on quartzite, sandstone and granite, which are silica-rich types of rocks, Fernandéz Verdía et al. (2000) noticed silica precipitation by microbial action in pores of these deposits forming portions with no empty spaces. The same authors attribute rhythmic accretion structures in concentric layers to biogenic processes. Similar features were identified in several samples in cavities of both Furnas and Vila Velha rocks which are therefore considered evidence of biologically induced mineralization (Fig. 15).
CONCLUSION

The speleothems formed on Furnas Formation and Vila Velha sub-unit quartz-sandstones present themselves in six types: (1) arborescent, (2) mushroom-shaped, (3) stalactitic, (4) stalagmitic, (5) columnar with horizontal/ascending growth, and (6) fibrous. The deposits like rimstones of the study area are more properly classified as clastic deposits since they bear no evidence of chemical precipitation, despite being considered speleothems in previous studies. Nonetheless, the possibility of mineral precipitation is not ruled out. Detail studies on outer portions of some deposits are therefore necessary, especially where rigid crusts are present.

Biosignatures, various bacteria forms (possibly cyanobacteria and Gallionella ferruginea), diatoms and other microorganisms capable of promoting precipitation of minerals such as silica, kaolinite and iron oxides were identified in several of the speleothems studied. Microbial action records are present in nearly all samples studied, suggesting biological influence on the genesis of speleothems. These secondary mineral deposits can herefore be classified as microbialites as their genesis involved biologically induced mineralization.

In the study area, arborescent, stalactitic, stalagmitic and columnar speleothems with horizontal/ascending growth bear evidence of both microbiological and inorganic processes in their genetic phases. Mushroom-shaped speleothems are the most properly classified as microbialites as microbial influence prevailed in their formation processes, while fibrous deposits may correspond to mineral accumulation under the influence of microbial metabolism. However, the rarity of these latter speleothems, found in only one place and in very small number, rendered additional analyses unfeasible. Further investigation is necessary based on new occurrences.

No pattern of speleothem occurrence seems to be related to specific stratigraphic or sedimentary aspects of quartz-sandstones in the study area. However, speleothems tend to develop in sites controlled by stratification and fracturing planes. Such discontinuities favor percolation of water inside rock bodies, thus contributing to the initial weathering, release and removal of ions and molecules from the surface of minerals, a fundamental cycle to the availability of raw material for the development of secondary chemical deposits.

These speleothems, which result from dissolution of quartz grains and kaolinitic cement, are important indicators of karst processes in Furnas Formation and Vila Velha sub-unit quartz-sandstones. The microbial action identified may also have exerted influence on the chemical weathering of mineral components. Corroded quartz grains in altered rocks suggest microbial action, mainly on pH modifications that render water alkaline setting ideal conditions for chemical weathering of quartz.

The speleothems found in the study area host an abundant diversity of biological, possibly fossil materials capable of preserving paleoecological and paleoenvironmental records of great scientific value. For hosting hundreds of caves and an expressive set of karst features, outcropping areas of the Furnas Formation and Vila Velha Sandstone can be considered a speleological province and a new Brazilian karst region known the Campos Gerais do Paraná one.

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Authorship statement: HSP designed, directed, collected the data, performed the measurements, and wrote the study; LAF, MSM, GBG, and LLM collected and analyzed the data, wrote and revised the manuscript.


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Pontes et al.


The COVID-19 pandemic, caused by the novel coronavirus SARS-CoV-2, has been responsible for over 650,000 deaths worldwide. Transmission of SARS-CoV-2 occurs primarily through airborne transmission or direct human contact, demonstrating the importance of social distancing measures and the use of face masks to prevent infection. Nonetheless, the persistence of coronavirus on surfaces means that disinfection is important to limit the possibility of contact transmission. In this paper, the potential for various surfaces in show caves to serve as sources for SARS-CoV-2 infection is examined. Given the isoelectric potential (pI) of SARS and SARS-like coronaviruses, it is likely that they are adsorbed via electrochemical interactions to (limestone) rock surfaces, where the high humidity, pH and presence of bicarbonate ions will quickly lead to inactivation. Nonetheless, show caves contain infrastructure made of other non-porous surfaces that are more permissive for maintaining coronavirus viability. The 423 antiviral products approved by the US Environmental Protection Agency (EPA) were curated into 23 antiviral chemistries, which were further classified based on their potential to be hazardous, impact cave features or ecosystems, and those compounds likely to have the minimum impact on caves. The results suggest that alcohols (70% ethanol), organic acids (citric and lactic acid) and dilute hypochlorite represent the best disinfectants for in-cave use on non-porous surfaces. These disinfectants are able to inactivate coronaviruses in <5 min with minimal impact to cave features and ecosystems.

INTRODUCTION

In December, 2019 a cluster of seven patients suffering from an unusual pneumonia were admitted to the Jin Yin-Tan Hospital in Wuhan, Hubei Province, China (Zhou et al., 2020). Samples from five of these patients were shown to contain a novel coronavirus, initially termed 2019-nCoV (Zhou et al., 2020). Subsequent sequencing of the genome of this novel coronavirus demonstrated its high degree of similarity to the virus responsible for the 2003 SARS outbreak, and the virus was renamed SARS-CoV-2 (CoV-ICTV, 2020). This novel coronavirus is responsible for a complex range of symptoms, characterized by a persistent dry cough and fever, which can rapidly progress to a severe disease (termed COVID-19) and death, particularly in patients aged >60 years (CDC Covid-19 Response Team, 2020; WHO, 2020).

The sustained, person-to-person spread of SARS-CoV-2, exacerbated by a high incidence of asymptomatic spread (James et al., 2020) has led to a global pandemic, with an excess of 16 million known infections and over 650,000 deaths worldwide (Dong et al., 2020), although serology studies suggest the actual number of cases may exceed this number by 25- to 50-fold (Bendavid et al., 2020; Wilson, 2020). In order to limit the spread of SARS-CoV-2, the US Centers for Disease Control and Prevention (CDC) have published a series of guidelines that include social distancing, frequent handwashing and the wearing of masks in public (for example, see: https://www.cdc.gov/coronavirus/2019-ncov/prevent-getting-sick/index.html). Within the United States, many states also implemented ‘stay at home’ orders, with re-opening occurring on a state-by-state timeline (Adolph et al., 2020; Courtemanche et al., 2020).

Among the many businesses that have been impacted by these ‘stay at home’ orders are commercial show caves, which allow the general public to visit caves under relatively safe conditions, including artificial lighting and walkways. Worldwide there are an estimated 1,500 show caves (Spate &
Spate, 2014), with over 300 caves in the US. These include commercial caves, along with federal and state managed operations. In a 2013 survey, the 142 commercial show caves of the US National Caves Association, demonstrated an economic impact of >$220 million in annual revenue (Petrick & Chen, 2013), while commercial caves managed by the US National Park Service generate almost $500 million (Cullinane Thomas & Koontz, 2020). With almost 20 million visitors annually, these show caves provide important educational and outreach activities related to the history, geology and protection of cave biota in the subsurface (Petrick & Chen, 2013).

Given the size of tour groups, relatively confined conditions and potentially limited ventilation of caves, reopening cave tours will require managers to adapt to challenging social distancing and personal protective equipment (PPE) recommendations (CDC, 2020). An added complexity is related to how to disinfect artificial cave infrastructure (such as handrails and doors handles), while limiting the impact to native ecosystems, which can be particularly sensitive to chemical input (Barton, 2015). Human coronaviruses are not novel, and it is estimated that coronaviruses have been circulating within the human population for over 1,000 years (Pyrc et al., 2006; Woo et al., 2009). Before the emergence of SARS-CoV-2, there were three circulating human coronaviruses (HCoV): two (HCoV-229E and HCoV-OC43) are responsible for up to 30% of common colds, while HCoV-NL63 is associated with respiratory diseases in children (van der Hoek et al., 2004). Given the ubiquitous nature of these viruses, coronavirus inactivation has historically been included in the evaluation of many commercial and household disinfectants.

While many commercial caves have adopted protocols to disinfect surfaces and equipment to prevent the spread of White-nose Syndrome in bats, these approaches (which were developed to inactivate fungal spores), are much more aggressive than are necessary to inactivate an enveloped coronavirus (Maris, 1990; Shelley et al., 2012). Indeed, exposure of SARS-CoV-2 to environmental conditions can lead to viral inactivation in as little as 3 hours, while coronaviruses are up to 500-times more sensitive to disinfectants as non-enveloped viruses (Maris, 1990; van Doremalen et al., 2020). Nonetheless, in order to ensure the safety of visitors to show caves, it is important to disinfect surfaces that are expected to be touched by visitors. This paper examines the known chemistry and sensitivity of human coronaviruses to disinfectants and provides a number of recommendations for effective disinfection, while limiting potential impact to the cave environment.

**MATERIALS AND METHODS**

To calculate the pI of coronaviruses, viral protein sequences were obtained from the National Center for Biotechnology Information (NCBI) protein database in translated FASTA format (https://www.ncbi.nlm.nih.gov/protein), including those for which whole viral particle pI have been determined empirically. These sequences included the enteric bovine coronavirus spike protein (Bovine_CoV; accession NP_150077), rat sialodacryoadenitis coronavirus spike protein (RCoV-SDAV; accession AAP97738), bat SARS-like betacoronavirus spike protein (BCoV-SARS; accession ATO98157), bat SARS-like betacoronavirus BtRs spike protein (BCoV-BtRs; accession QDF43825), bat SARS-like betacoronavirus RaTG13 spike protein (BCoV-RaTG13; accession QHR63300), human betacoronavirus spike protein (HCoV-OC43; accession YP_009555241), human betacoronavirus SARS-CoV 2003 Urbani isolate spike protein (SARS-CoV; accession AAP13441), human betacoronavirus SARS-CoV-2 type isolate Wuhan-Hu-1 spike protein (SARS-CoV-2 Wuhan-Hu-1; accession YP_009724390), human betacoronavirus SARS-CoV-2 Australian isolate spike protein (SARS-CoV-2 Australian; accession QJR88869), human betacoronavirus SARS-CoV-2 Jamaican isolate spike protein (SARS-CoV-2 Jamaica; accession QJX70646), human betacoronavirus SARS-CoV-2 New York City isolate spike protein (SARS-CoV-2 CDC/USA; accession QJJ27937), 1918 influenza A H1N1 hemagglutinin (1918 Influenza H1N1; accession AAD17229), 2015 influenza A H5N8 hemagglutinin (2015 Influenza H5N8; accession ALB07770), 2009 swine influenza A H1N1 hemagglutinin (2009 Swine Flu H1N1; accession ACDD8516), rabies lyssavirus wolf isolate surface glycoprotein (Rabies; accession AJP77607), human poliovirus 1 strain Sabin VP0 polyprotein (human poliovirus 1 strain Sabin VP0 polyprotein (accession CAA24465). The isoelectric point was calculated using the IPC isoelectric point calculator, which uses 16 different algorithms to calculate pI (Kozlowski, 2016). The average pI was plotted versus protein molecular weight (kDa) in Excel and imported into Adobe Illustrator 2020 to create the final plot.

The US Environmental Protection Agency (EPA) List N registry of disinfectants for use against SARS-CoV-2 was used to identify antiviral disinfectants based on active ingredient (https://cfpub.epa.gov/gwiz/disinfectants/index.cfm). The activity of each compound against coronaviruses or enveloped viruses in general was based on established antiviral activity, with human health impact determined by cross-referencing to a toxicology database (Poli et al., 1979; McDonnell & Russell, 1999; Wolff et al., 2005; Bateman et al., 2014; Kampf et al., 2020).

**RESULTS**

To date the majority of SARS-CoV-2 infections in humans occur through direct human contact or through respiratory droplets and aerosols (Prather et al., 2020; Santarpia et al., 2020; Stadnytskyi et al., 2020). Nonetheless, the detection of SARS-CoV-2 on surfaces means that they can provide a potential source for contact transmission, particularly in the case of non-porous surfaces, such as plastics, glass and metal (Sattar & Sprinthorpe, 1996; Wolff et al., 2005; Dietz et al., 2020; van Doremalen et al., 2020; Santarpia et al., 2020). The likelihood of porous surfaces, such as the host rock (limestone) of the cave to serve as a potential surface for contact transmission
was examined by comparing the known geochemistry of calcite with coronavirus particle chemistry.

Broadly, the isoelectric point (pl) is a measure of average charge. While the pl is generally calculated or measured for individual proteins to determine the strength of electrostatic interactions, it has also been used to determine the sorption characteristics of whole virus particles (virions) to mineral surfaces (Miller et al., 1944; Carlson Jr et al., 1968; Moore et al., 1981). In this study, the pl of coronaviruses, SARS-like coronaviruses and SARS-CoV/SARS-CoV-2 surface proteins were compared to other enveloped RNA viruses where empirical pl values also exist (Fig. 1). In the case of influenza A virus, the pl was calculated based on the dominant surface protein, hemagglutinin, to obtain a value of 6.0 - 6.8 depending on viral strain, which included the source of the 1918 Spanish flu pandemic, the 2009 swine flu pandemic, and a 2015 bird flu (Fig. 1). As influenza includes other surface proteins along with a negatively charged phospholipid bilayer, this obtained value was compared to the measured pl for the entire viral particle, which is 5.0 to 5.9 (Miller et al., 1944; Vajda et al., 2016). Similar to coronaviruses, rabies has a dominant glycoprotein with a calculated pl of 7.6 (Fig. 1), which has been measured as pl 7.0 for the whole viral particle (Atanasiu et al., 1979). Therefore, while the dominant surface proteins cannot give a net surface charge for the whole virus particle, the pl values likely underestimate the net negative charge of the virus by 0.5 - 1.0 pl units (Lakshminarayanaiah & Murayama, 1975; Pekker & Schneider, 2015).

**Fig. 1.** Calculated isoelectric point (pl) values versus molecular weight for the dominant surface proteins of enveloped RNA viruses, including coronaviruses, influenza A and rabies. The average calculated pl is plotted for these viruses, along with the measured, whole virion particle pl values for influenza A and rabies.

For coronaviruses, the pl of the spike protein was determined for animal coronaviruses (bovine and rat), along with circulating human betacoronavirus (HCoV-OC43), the SARS-like bat coronaviruses (BCoV_RaTG13, BCoV-SARS and BCoV-BtRs) and human SARS viruses (SARS-CoV/SARS-CoV-2). In the case of SARS-CoV-2, representative virus spike proteins were used from four geographically separate isolates (Fig. 1). The data indicate a consistent clustering of the coronaviruses with a pl between 4.9 and 6.1, although as stated, due to membrane chemistry, this value is likely to be lower for the whole virus particle (Pekker & Shneider, 2015). These data suggest that the SARS and SARS-like coronaviruses have a net negative charge, which will result in a strong electrostatic attraction between the virus and calcium carbonate surfaces, such as limestone and concrete.

There are a number of artificial, non-porous surfaces (plastics, metal and glass) in show caves that form the tour infrastructure (i.e., handrails and door handles). Given that coronaviruses can persist longer on these surfaces, especially at cooler temperatures and in the absence of sunlight, these surfaces should be disinfected to limit the possibility of contact spread (Wolff et al., 2005; Kampf et al., 2020; Ratnesar-Shumate et al., 2020). An evaluation of the 423 products recommended by the US EPA for use against SARS-CoV-2 was carried out. This multitude of products was reduced to 27 antiviral chemistries (Table 1), of which four were removed from further consideration: the antimicrobial activity of sodium chloride depends on its interaction with hypochlorous acid to produce sodium hypochlorite, sodium carbonate is used to stabilize sodium hypochlorite, and ammonium carbonate and bicarbonate are used as pH neutralizing agents (McDonnell & Russell, 1999). The remaining 23 antiviral chemistries were further grouped into four categories for the potential of in-cave use based on potential impact descriptions: 1) potentially toxic under cave conditions; 2) chemical impact; 3) reduced impact; and 4) lowest impact (Table 1). As a reference, mechanism of action in viral inactivation (mechanism), effective concentrations (active concentration) and contact time for antiviral activity (contact time) by these chemistries are shown.

**Potentially toxic**

A number of the oxidants recommended by the EPA should be avoided in cave settings due their strong biocidal activities, which can either negatively affect
Table 1. Approved EPA compounds with antiviral activity against human coronaviruses.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Chemistry</th>
<th>Mechanism</th>
<th>Active Conc</th>
<th>Contact Time</th>
<th>Usage</th>
<th>Example Brand$^1$</th>
<th>Contact Time</th>
<th>Wipe Conc$^4$</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorine dioxide</td>
<td>ClO$_2$</td>
<td>Oxidant</td>
<td>0.02%</td>
<td>1 min</td>
<td>I, R</td>
<td>Electro-Biocide</td>
<td>10 min</td>
<td>-</td>
<td>Lim, 2010$^1$</td>
</tr>
<tr>
<td>Peroxyacetic acid</td>
<td>CH$_3$COOOH</td>
<td>Oxidant</td>
<td>0.20%</td>
<td>10 min</td>
<td>I, R</td>
<td>Maguard 1522</td>
<td>1 min</td>
<td>-</td>
<td>Becker, 2017$^1$</td>
</tr>
<tr>
<td>Potassium peroxymonosulfate</td>
<td>KOHSO$_4$</td>
<td>Oxidant</td>
<td>1%</td>
<td>1 min</td>
<td>I, R</td>
<td>Virkon S$^+$</td>
<td>10 min</td>
<td>-</td>
<td>Wolff, 2005</td>
</tr>
<tr>
<td>Sodium carbonate peroxide</td>
<td>Na$_2$CO$_3$·1.5H$_2$O$_2$</td>
<td>Oxidant</td>
<td>500 ppm</td>
<td>60 min</td>
<td>I, R</td>
<td>Sterilex Ultra Powder</td>
<td>10 min</td>
<td>-</td>
<td>Nakano, 1998</td>
</tr>
<tr>
<td>Sodium dichloroisocyanurate</td>
<td>C$_3$Cl$_2$N$_3$NaO$_3$</td>
<td>Oxidant</td>
<td>500 ppm</td>
<td>2 min</td>
<td>I, R</td>
<td>Klorosept</td>
<td>1 min</td>
<td>-</td>
<td>Bloomfield, 1990</td>
</tr>
<tr>
<td>1,2-Hexanediol</td>
<td>(CH$_2$CH$_2$OH)$_2$</td>
<td>Alcohol</td>
<td>-</td>
<td>-</td>
<td>I</td>
<td>PELS 422$^1$</td>
<td>10 min</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Glutaraldehyde</td>
<td>C$_2$H$_4$O$_2$</td>
<td>Protein crosslinking</td>
<td>8%</td>
<td>15 s</td>
<td>I</td>
<td>Synergize</td>
<td>10 min</td>
<td>7%</td>
<td>Kampf, 2020</td>
</tr>
<tr>
<td>Hydrogen peroxide</td>
<td>H$_2$O$_2$</td>
<td>Oxidant</td>
<td>0.50%</td>
<td>3 min</td>
<td>I</td>
<td>SynerSys$^+$</td>
<td>5 min</td>
<td>-</td>
<td>Wolff, 2005</td>
</tr>
<tr>
<td>Quaternary ammonium</td>
<td>R$_4$N$^+$</td>
<td>Membrane disruption</td>
<td>0.10%</td>
<td>5 min</td>
<td>I, R</td>
<td>Sani-cloth Germicidal</td>
<td>3 min</td>
<td>0.23%</td>
<td>Wolff, 2005</td>
</tr>
<tr>
<td>Hydrochloric acid (liquid/gas)</td>
<td>HCl</td>
<td>pH</td>
<td>7%</td>
<td>1 min</td>
<td>I, R</td>
<td>Lysol Brand Toilet Bowl Cleaner</td>
<td>10 min</td>
<td>-</td>
<td>Sattar, 1989</td>
</tr>
<tr>
<td>Phenol</td>
<td>C$_6$H$_5$OH</td>
<td>Membrane disruption</td>
<td>0.02%</td>
<td>5 min</td>
<td>I, R</td>
<td>Sporicidin Towelettes</td>
<td>5 min</td>
<td>1.56%</td>
<td>Wolff, 2005</td>
</tr>
<tr>
<td>Triethylene glycol</td>
<td>HOCH$_2$CH$_2$OCH$_2$CH$_2$OH</td>
<td>Alcohol</td>
<td>50 ppm</td>
<td>2.5 min</td>
<td>R</td>
<td>Scrubbing bubbles</td>
<td>5 min</td>
<td>-</td>
<td>Rudnick, 2009$^§$</td>
</tr>
<tr>
<td>Dodecylbenzenesulfonic acid</td>
<td>C$<em>{12}$H$</em>{25}$C$_6$H$_4$.SO$_3$H</td>
<td>Membrane disruption</td>
<td>0.1%</td>
<td>5 min</td>
<td>I, R</td>
<td>Ecolab CW32A</td>
<td>0.5 min</td>
<td>-</td>
<td>Jean, 2003$^†$</td>
</tr>
<tr>
<td>Silver (ions)</td>
<td>Ag$^+$</td>
<td>Protein inactivation</td>
<td>250 ppm</td>
<td>30 min</td>
<td>I, R</td>
<td>Axen 30</td>
<td>3 min</td>
<td>-</td>
<td>Minoshima, 2016</td>
</tr>
</tbody>
</table>

Notes:
- $^1$ EPA recommendations
- $^2$ Chemical impact
- $^3$ Potentially toxic under cave conditions
- $^4$ Refers to the specific reference for each compound.
<table>
<thead>
<tr>
<th>Impact</th>
<th>Disinfectant</th>
<th>Chemical formula</th>
<th>Effect</th>
<th>Time</th>
<th>Use</th>
<th>Product</th>
<th>Duration</th>
<th>Concentration</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reduced impact</td>
<td>Thymol</td>
<td>CH(CH$_3$)$_2$CH$_2$OH</td>
<td>Membrane disruption</td>
<td>2%</td>
<td>2 h</td>
<td>I, R</td>
<td>10 min</td>
<td>0.10%</td>
<td>Sanchez, 2015</td>
</tr>
<tr>
<td></td>
<td>Hypochlorous acid</td>
<td>HClO</td>
<td>Oxidant</td>
<td>150 ppm</td>
<td>1 min</td>
<td>I, R</td>
<td>10 min</td>
<td>-</td>
<td>Chander, 2012</td>
</tr>
<tr>
<td></td>
<td>Sodium chlorite</td>
<td>NaClO$_2$</td>
<td>Oxidant</td>
<td>0.05%</td>
<td>1 min</td>
<td>I</td>
<td>10 min</td>
<td>-</td>
<td>Kampf, 2020</td>
</tr>
<tr>
<td></td>
<td>Sodium hypochlorite</td>
<td>NaClO</td>
<td>Oxidant</td>
<td>0.20%</td>
<td>5 min</td>
<td>I, R</td>
<td>1 min</td>
<td>0.65%</td>
<td>Wolff, 2005</td>
</tr>
<tr>
<td>Lowest impact</td>
<td>Citric acid</td>
<td>C$_6$H$_8$O$_7$</td>
<td>Acid</td>
<td>0.20%</td>
<td>10 min</td>
<td>I, R</td>
<td>5 min</td>
<td>0.60%</td>
<td>Krug, 2011</td>
</tr>
<tr>
<td></td>
<td>Ethanol</td>
<td>C$_2$H$_5$OH</td>
<td>Alcohol</td>
<td>70%</td>
<td>30 s</td>
<td>I, R</td>
<td>1 min</td>
<td>70%</td>
<td>Kampf, 2020</td>
</tr>
<tr>
<td></td>
<td>Glycolic acid</td>
<td>CH$_2$COCOOH</td>
<td>Acid</td>
<td>5%</td>
<td>10 min</td>
<td>R</td>
<td>10 min</td>
<td>-</td>
<td>Poli, 1978</td>
</tr>
<tr>
<td></td>
<td>Isopropanol</td>
<td>C$_3$H$_7$OH</td>
<td>Alcohol</td>
<td>70%</td>
<td>30 s</td>
<td>I</td>
<td>3 min</td>
<td>70%</td>
<td>Kampf, 2020</td>
</tr>
<tr>
<td></td>
<td>Lactic acid</td>
<td>CH$_3$CHOHCOOH</td>
<td>Acid</td>
<td>5%</td>
<td>10 min</td>
<td>I, R</td>
<td>5 min</td>
<td>-</td>
<td>Poli, 1978</td>
</tr>
</tbody>
</table>

Information compiled from [https://cfpub.epa.gov/giwiz/disinfectants/index.cfm](https://cfpub.epa.gov/giwiz/disinfectants/index.cfm)

- = I:Certified for institutional or R: residential use;
† = Determined on non-enveloped viruses;
‡ = EPA approval only, no published details;
§ = Data published for air disinfection by gas;
|| = Where possible, identified products were wipes;
# = Concentration of active ingredient in identified wipes;
** = Products for which MSDS sheets warn about soil interactions;
endemic species (and human visitors), or are highly corrosive (McDonnell & Russell, 1999). Indeed, many of these compounds are only recommended for use in healthcare or industrial settings due to such safety considerations. Of these oxidants, chlorine dioxide can produce a poisonous gas at room temperature, and even at low concentrations is a strong irritant of mucous membranes (Bateman et al., 2014). Similar oxidants include peroxyacetic acid, potassium peroxymonosulfate, sodium corbanote peroxhydurate, and sodium chlorisocyanurate (McDonnell & Russell, 1999; Bateman et al., 2014). While glutaraldehyde and 1,2-hexanediol are effective disinfectants at low concentrations, without adequate ventilation (as would be expected in cave environments) they have the potential to be strong irritants (Bateman et al., 2014). Finally, while hydrogen peroxide breaks down into the relatively inert oxygen and water, it is highly corrosive and reacts with iron in the Fenton reaction to generate dangerous hydroxyl radicals that cause severe damage in biological molecules, and DNA in particular (Rafat Husain et al., 1987; Winterbourn, 1995). Cave sediments commonly contain iron-oxides (comprised of goethite and ferrihydrite) as a residue of past cave-forming processes, which give cave sediments and muds their characteristic brown-to-red coloration (Palmer, 2007; White, 2007; Blatnik et al., 2020). Indeed, these iron-rich deposits have proven invaluable in paleomagnetic studies to age many cave features (Luizser, 2009; Blatnik et al., 2020). The average iron content of cave sediments has been estimated to range between 0.1–2.0% (Sasowsky & Mylroie, 2004; Sasowsky, pers. comm., 2020), and given that Fe(III) can be recycled to Fe(II) by microbial activity in cave sediments, this suggests that even trace iron in the environment is sufficient to catalyze the Fenton reaction (Schwertmann, 1991; Parker et al., 2018).

### High impact

Several of the compounds recommended for disinfection have the potential for a significant impact on the cave environment, either through interactions with host rock chemistry or microbial ecosystems. With regard to host rock chemistry, hydrochloric acid is an aggressive acid that readily dissolves limestone and is particularly problematic in regard to speleothem damage. Caves are also nutrient-limited environments, particularly in regard to nitrogen (Barton, 2015), and while a number of ammonium-based compounds are likely to be safe and effective, the introduction of excess nitrogen can be particularly detrimental to microbial system dynamics (Barton, 2015; Hershey & Barton, 2018). A notable example of such impact is in Lascaux Cave, where introduced quaternary ammonium compounds (quats) has resulted in excessively high nitrogen content in cave sediments, dramatically altering the normal microbiology of the cave and leading to damage to the important Neolithic paintings (Bastian et al., 2009). Similarly, the low levels of organic carbon found in caves (<0.5 mg/L) makes them sensitive to organic input (Barton, 2015). A number of the recommended disinfectants are based on reactive aromatic and long-chain hydrocarbons (including phenol, triethylene glycol, and dodecybenzenesulfonic acid), which are likely to introduce unreasonably high levels of organic carbon contamination (Barton, 2015). Finally, while transition metals are common trace elements in limestone, silver ions (which have strong antimicrobial activity) are not naturally broken down, leading to extended contamination in sediments and resultant changes in microbial community structure (Klaine et al., 2008).

### Reduced impact

The EPA disinfectant list contains a number of effective compounds that are likely to have other, undesired consequences. Among these is thymol, which has a strong, antiseptic odor, while a 1:10 solution of household bleach (hypochlorite), sodium chlorite or hypochlorous acid (which dissociate to form hypochlorite) have a strong chlorine smell that can be intense without adequate ventilation. Thus, while these compounds are safe and effective at low concentrations, their use may be limited simply through the tolerance of visitors and staff.

### Lowest impact

The EPA list contains a number of antimicrobial agents that have chemistries similar to organic compounds naturally found in caves, including citric and lactic acid (Bullen et al., 2008). These compounds are made during microbial metabolic processes (overflow metabolism) and include alcohols, allowing them to be easily broken down by native microbial communities (Xu et al., 1999; Vemuri et al., 2006); however, as with other organic carbon, their excessive use will alter microbial community structure (Barton, 2015; Hershey & Barton, 2018). Such compounds also have the least likelihood of being problematic if tourists were to pick them up on their hands, for example, citric acid is found in oranges, while ethanol and isopropanol quickly volatilize into the atmosphere.

### DISCUSSION

One of the concerns regarding COVID-19 in caves was the potential of SARS-CoV-2 to survive long-term under the temperature, humidity and low light conditions of caves, including whether the rock surfaces could serve as a source of possible transmission (R. Toomey, personal communication, 2020). While there are currently no studies that have specifically examined the persistence of coronaviruses in caves, these viruses have been tested under a number of conditions that mimic the cave environment. Human coronaviruses (HCoV-229E and OC43) and SARS-CoV have been shown to rapidly lose infectivity in high humidity (>50%) (Kim et al., 2007; Chan et al., 2011; Dietz et al., 2020). This inactivation is believed to occur through two processes: 1) high humidity prevents the dehydration of droplets to reduce particle size, which is necessary for the virus to remain buoyant and promote person-to-person spread (Stadnytskyi et al., 2020); and 2) high humidity destabilizes the polar residues

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in the viral envelope, which is critical to maintaining viral infectivity (Yang & Marr, 2012). Given the high humidity of caves environments (>95%), this suggests rapid inactivation will occur (Wigley & Brown, 1976). Nonetheless, coronaviruses do persist longer at cooler temperatures and in the absence of sunlight, and the impact of high relative humidity and geochemistry should not be considered an inherent inactivation approach that negates the need to disinfect surfaces that visitors touch (Ratnesar-Shumate et al., 2020; van Doremalen et al., 2020).

A significant amount of work has explored virus adsorption and inactivation in mineral substrates, particularly for poliovirus, which was historically a dangerous groundwater pathogen (Poli et al., 1979; Murray & Parks, 1980; Moore et al., 1981). Poliovirus has a calculated capsid pl of 6.4 and a measured pl of 5.9, similar to that of coronaviruses (Carlson Jr et al., 1968; Murray & Parks, 1980). The anionic surface charge (net negative charge) of the virus predicts how the particle will interact and adsorb to surfaces, which is governed by general electrodynamic potentials (Murray & Parks, 1980). The surface charge of limestone is derived from the potential of the ions in solution on the surface of the rock, which is dominated by the cation Ca²⁺ (although HCO₃⁻, CO₃²⁻, H⁺ and OH⁻ ions contribute to surface chemistry), and has been empirically shown to have a pl between 8.0 to 9.5, with commonly measured pH values of 8.3 (Somasundaran & Agar, 1967; Banks et al., 2010). This net positive charge will strongly attract negatively charged coronavirus particles, with electrochemical interactions causing strong adsorption through hydrogen bonding and van der Waals forces (Murray and Parks, 1980). The stronger the difference in electrochemical charge between surfaces (with pl as a proxy) the stronger this attraction, which will outweigh the impact of pH (Carlson Jr et al., 1968; Murray and Parks, 1980; Moore et al., 1981). Indeed, strong poliovirus attraction has been demonstrated on positively charged iron oxide and dolomite surfaces, indicating that limestone surfaces and iron-rich cave sediments are capable of binding high levels of SARS-CoV-2 particles (Murray & Parks, 1980; Moore et al., 1981). Further, bicarbonates and high pH (>8.0) readily inactivate coronaviruses, both of which are satisfied on limestone surfaces (Moore et al., 1981; Lamarre & Talbot, 1989).

Past work on disinfection in cave environments have been focused on WNS disinfection or the removal of algal and cyanobacterial growth around artificial lighting (lampenflora; Boston, 2006; Mulec & Kosi, 2009; Shelley et al., 2012; Meyer et al., 2017). Among the disinfectants identified for use against SARS-CoV-2, only quaternary ammonium compounds and hypochlorite were recommended for WNS disinfection protocols (Table 1), while hypochlorite and high concentrations of hydrogen peroxide (15 wt/%) are recommended for lampenflora (Shelley et al., 2012; Meyer et al., 2017). Enveloped viruses are much more sensitive to inactivation than fungal spores or algal cysts and such aggressive approaches to disinfection are unnecessary (Maillard, 2001). Indeed, enveloped viruses such as coronaviruses are 500-times more sensitive to hypochlorite than non-enveloped viruses, and are also sensitive to a range of chemistries that would have no impact on WNS or algae, including weak acids, alkalis, or alcohol-dehydration (Table 1; Poli et al., 1979; Wolff et al., 2005; Kampf et al., 2020). This provides a variety of compounds that have the potential to inactive coronaviruses on surfaces and prevent contact transmission, as indicated by the 423 products identified by the EPA.

### Special consideration on the use of hydrogen peroxide

Past researchers have recommended the use of hydrogen peroxide in caves, due to its lack of odor and ability to break down into inert H₂O and O₂ (Boston, 2006; Boston et al., 2006; Mulec & Kosi, 2009); however, in the presence of reduced or oxidized iron, the Fenton reaction produces hydroxyl radicals (Eq. 1) and other reactive species (including hydroxides and hydroperoxyls; Eq. 2):

\[ \text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{HO}^- + \text{OH}^- + \text{H}^+ \]  
(1)

\[ \text{Fe}^{3+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{2+} + \text{HOO}^- + \text{H}^+ \]  
(2)

The reactive species are dangerous to biological systems, causing extensive DNA damage and mutagenesis, even at pM concentrations (Winterbourn, 1995). This activity can be accentuated in cave environments by the microbially-enhanced dissolution of iron oxides, which is promoted under the mineral grain size and chemistry of iron-rich sediments similar to those in caves (Schwertmann, 1991; Parker et al., 2018). Indeed, in the past, researchers who have demonstrated the successful use of hydrogen peroxide to remove lampenflora, only did so when high concentration solutions (>15%) were used, which required the use of protective respirators and goggles given the poor ventilation in caves and noxious gases produced (Meyer et al., 2017). Given the extreme reactivity and corrosive activity of hydrogen peroxide (which can attack limestone surfaces as well as metals; Mulec & Kosi, 2009) and given that even trace levels of hydroxyl radicals can be acutely toxic (one of the reasons human cells have such effective iron-scavenging mechanisms is limit the Fenton reaction from the tiny amounts of H₂O₂ generated during cellular respiration; Barbouti et al., 2001; Imlay, 2008), extreme caution should therefore be used if choosing to use hydrogen peroxide in caves, as highlighted by the MSDS sheets for these products: when compiling the MSDS data for Table 1, only the strong oxidant potassium peroxymonsulfate (Virkon S) and hydrogen peroxide (SynerSys) contain explicit warnings to avoid interaction with soils (for example, compare [http://www.fourupack.com/myfile.aspx?doc=48328_SynerSysSporidicalDisinfectantP ART_A_and_B_SDS.pdf%7c2](http://www.fourupack.com/myfile.aspx?doc=48328_SynerSysSporidicalDisinfectantPART_A_and_B_SDS.pdf%7c2) and [https://media3. cleanfreak.com/documents/sds/lysol-all-purpose-cleaner-with-bleach.pdf](https://media3. cleanfreak.com/documents/sds/lysol-all-purpose-cleaner-with-bleach.pdf). Nonetheless, all of the chemicals on the EPA List N are effective against coronaviruses and hydrogen peroxide may be appropriate for use on glass or plastic surfaces if their reaction with cave sediments is prevented.
CONCLUSIONS

To date, there are no recorded cases of COVID-19 transmission have been identified as the result of surface contact transmission (Dietz et al., 2020) and the majority of cases can be traced to airborne transmission or direct human contact, demonstrating the importance of social distancing measures and the use of face masks (Prather et al., 2020; Santarpia et al., 2020; Stadnytskyi et al., 2020). The calculated pH of SARS-CoV-2 suggests that in caves the virus would be adsorbed onto limestone surfaces, further reducing the likelihood that these surfaces could serve as a source of virus transfer (Fig. 1). In addition, the high humidity of caves and chemistry of limestone surfaces (including inactivating bicarbonates and high pH) are likely to rapidly inactivate adsorbed coronavirus particles, suggesting that the limestone/rock surfaces or concrete walkways pose no specific danger for contact transmission. Nonetheless, non-porous surfaces (such as plastics, metals and glass) are more permissive to coronavirus viability and tourist operators should focus on disinfection of infrastructure from these materials that visitors will touch. Given the 423 antiviral agents recommended by the EPA, the following compounds are recommended for use in caves (Table 1):

Alcohols: Ethanol is naturally produced by microbial metabolism and has been shown to sorb to calcium carbonate, suggesting it is common in caves (Bullen et al., 2008). An effective concentration of 70% ethanol will inactivate coronaviruses by dehydration in 5 min (Wolff et al., 2005).

Organic acids: The viral cell envelope of coronaviruses is particularly sensitive to destabilization by acids (Poli et al., 1979; Lamarre & Talbot, 1989). These organic acids, such as citric acid, share similarity in chemistry to organic acids that are naturally found in caves (Bullen et al., 2008).

Hypochlorite: While hypochlorite can produce a chlorine smell, even at low concentrations hypochlorite is one of the most effective disinfectants against coronaviruses (Maillard, 2001): a 1:50 dilution of household bleach (final concentration 0.12%) will inactivate coronaviruses in <5 min (Maillard, 2001; Wolff et al., 2005). While chlorine ions are likely to impact cave fauna (Boston, 2006), a study using 0.5% HOCl to treat lampenflora (which is higher than the recommended 0.1% to inactivate coronaviruses) demonstrated no increased impact on a cave adapted indicator species (the springtail Tomocerus celsius; Meyer et al., 2017). Others have suggested that hypochlorite leaves an oxidizing residue within caves (Boston et al., 2006). The reaction of hypochlorite with limestone (Eq. 3):

$$\text{CaCO}_3 + 2\text{NaOCl} \rightarrow \text{Na}_2\text{CO}_3 + \text{Ca(ClO)}_2$$

produces sodium bicarbonate and calcium hypochlorite. While calcium bicarbonate is fairly innocuous (and is a common food additive), calcium hypochlorite is more reactive and is used as a whitening agent in laundry detergent (it is identified as a white, powdery residue that smell weakly of bleach when scratched). Under acidic conditions, calcium hypochlorite is protonated to hypochlorous acid, which is a strong oxidant; however, limestone buffers the surface chemistry to an alkaline pH 8.3. Thus, while care should be taken to avoid generating conditions that produce high concentrations of hypochlorite in caves, the underlying chemistry of bleach in caves is preferable to the nitrogen introduction of quats and much safer than the potential damage of hydrogen peroxide to human health.

In recommending these compounds for in-cave use, it should be noted that all disinfectants have the ability to negatively impact cave microbiota (Estévez et al., 2019). To limit the unnecessary introduction of these compounds into caves, the lowest concentration necessary to inactivate SARS-CoV-2 should be used (Table 1), as increased concentrations provide no additional antiviral activity, and in the case of alcohols, higher concentrations actually have reduced antiviral activity. To limit impact, disinfecting wipes have a number of advantages over the use of sprayed liquid disinfectants, these include: 1) stabilization of disinfection chemistry in wipes means that they are less-likely to produce aerosols or off gas into the environment, particularly in the case of flammable substrates (Tyan et al., 2019); 2) wipes contain disinfectants that have already been diluted to the working concentration on an appropriate adsorbant, reducing the likelihood of excessive chemical introduction; 3) wipes are convenient to carry into the caves (including on tours) and are unlikely to unnecessarily introduce chemicals or organics to the environment through spraying or accidental spills; and 4) the use of wipes are unlikely to leave excessive residues on surfaces.

The recommended disinfectants in Table 1 have been curated to identify those available as wipes, including the advised contact time for coronavirus inactivation and concentration of active ingredient (derived from the MSDS) for each chemistry when compared to published laboratory inactivation studies. In many cases, due to the underlying chemistry, wipes may not appropriate (Table 1), are unavailable during the epidemic, or may not represent a cost-effective approach to disinfection. In this case, the full list of EPA database, which is searchable using active ingredients, should be used to identify alternatives (https://www.epa.gov/pesticide-registration/list-n-disinfectants-use-against-sars-cov-2). Regardless of choice, these procedures are suggested to limit the potential for contact transmission of SARS-CoV-2 in a way that minimizes impact on the cave ecosystem; however, given the generally poor ventilation in caves, they in no way negate the continued need for social distancing and face masks to limit transmission of this dangerous virus.

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Authorship statement: The author collected the data, carried out the analyses and wrote the manuscript.

REFERENCES


Did you wash your caving suit? Cavers’ role in the potential spread of *Pseudogymnoascus destructans*, the causative agent of White-Nose Disease

Violeta Zhelyazkova 1, Antoniya Hubancheva 2, Georgi Radoslavov 2, Nia Toshkova 1, and Sebastien J. Puechmaille 3*

1National Museum of Natural History, Bulgarian Academy of Sciences, 1 Tsar Osvoboditel Str., 1000 Sofia, Bulgaria
2Institute of Biodiversity and Ecosystem Research, Bulgarian Academy of Sciences, 25 Academic Georgi Bonchev Str, 1113 Sofia, Bulgaria
3ISEM, University of Montpellier, CNRS, EPHE, IRD, Montpellier, France

Abstract: White-Nose Disease (WND) has killed millions of hibernating bats in the US and Canada. Its causative agent, the fungus *Pseudogymnoascus destructans* was introduced to North America, but is native to Europe and Asia, where it is not associated with mass mortality. Although it is nearly impossible to eradicate an emerging wildlife disease, research on *P. destructans* spread mechanisms can aid in prevention of new introductions and development of better environmental management strategies. It is of particular importance to quantify the potential role of people visiting caves (cavers, tourists, bat researchers, etc.) whom inadvertently move *P. destructans* spores between sites, and to limit spread, particularly to areas where the fungus is absent. In the course of two consecutive field seasons, samples were collected from the equipment before and after work in Bulgarian caves where *P. destructans* is present. Viable *P. destructans* spores were isolated from field equipment after nearly 100% of cave visits, irrespective of the season. Results from lab experiments show the pathogen’s spores can remain viable on pieces of contaminated caving equipment under room temperature for at least 25 days, with no significant reduction in germination rates. This is concerning evidence indicating the potential for movement of viable *P. destructans* spores across countries and continents. Results further demonstrated that any type of regular washing can successfully remove the majority of *P. destructans* spores from several fabric types. Brushing complemented by washing in a ‘washing machine’ was slightly more effective than brushing alone, while a washing temperature of 50°C was more effective than temperatures of 30 and 40°C. However, none of the methods herein tested fully removed *P. destructans* and hence they are not a substitute for decontamination. Since many cavers forgo washing equipment after surveys, and bat workers often visit several underground sites per day during monitoring activities without cleaning equipment in between sites, it is essential to raise awareness on this subject and introduce hygienic protocols for the prevention of pathogen pollution in underground sites.

Keywords: WNS, bats, cave management, emerging infectious diseases, pathogen pollution

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INTRODUCTION

Introduction of non-native pathogens into previously unoccupied areas, known as ‘pathogen pollution’ (Cunningham et al., 2003) is a serious threat to wildlife, and has the potential to cause emergent infectious diseases and local extinctions (e.g., Daszak et al., 2000; De Castro & Bolker, 2005). Such is the case of White-Nose Disease (WND) in hibernating bats, responsible for one of the greatest mammalian mass mortalities in modern history (Puechmaille et al., 2011a; Frick et al., 2015). In line with the increasing rate of emergent fungal infections worldwide (Fisher et al., 2012), its causative agent is a fungus: the cave-dwelling species *Pseudogymnoascus destructans* (Lorch et al., 2011; Warnecke et al., 2012). The pathogen is considered native to Eurasia (Leopardi et al., 2015; Drees et al., 2017b) where it is not associated...
with mass morbidity or mortality (Puechmaille et al., 2010; Puechmaille et al., 2011b; Zukal et al., 2016; Fritz & Puechmaille, 2018; Kovacova et al., 2018) and was only recently introduced to North America. First documented in 2006 in New York State (Reeder & Turner, 2008; Veilleux, 2008), it is now confirmed in 38 U.S.A states and seven Canadian provinces, and estimated to have caused between 5.5 and 6.7 million bat deaths (U.S. Fish and Wildlife Service, 2020), thus significantly affecting both natural and agricultural ecosystems.

At short distances, bats migrating between roosts seem to be the main vector of *P. destructans* but long-distance dispersals (for instance, from Eastern to Western North America or from Europe to North America) are suspected to be human-mediated (Petit & Puechmaille, 2015; Lorch et al., 2016). As *P. destructans* not only colonizes bat skin, its long term persistence in the hibernacula environment (Puechmaille et al., 2011b; Raudabaugh & Miller, 2013; Reynolds et al., 2015; Fischer et al., 2020) may cause humans visiting underground sites to become potential vectors via their ‘dirty’ clothes and equipment. For example, Ballmann et al. (2017) reported the presence of *P. destructans* DNA on equipment used at WND-impacted sites and Reynolds & Barton (2014) pointed out more than $1 \times 10^6$ fungal spores (across species) can be found on human footwear after a single cave visit. Additionally, Campbell et al. (2020) showed *P. destructans* can survive at temperatures above 20°C and be potentially transported by bats outside of cave hibernacula. However, no research has attempted to look at the frequency of occurrence in human-mediated spread of *P. destructans*.

Given the serious impact of WND to bat populations, related instructions for cavers’ behaviour based on laboratory experiments (e.g., Puechmaille et al., 2011c; Shelley et al., 2013) were developed shortly after the disease outbreak in North America (the term cavers is used here for people utilizing caves/mines/underground sites; including bat researchers). National Decontamination Protocols adopted by the USA and Canada (U.S. Fish and Wildlife Service, 2020) advise ‘all persons who come into contact with bats, their environments, and/or associated materials’ to take precautions such as site-dedication of clothing and equipment, washing with laundry detergents and/or at temperatures above 50°C, or disinfection using, alcohol, hydrogen peroxide, or chlorine bleach solution products. As a preventive strategy, cavers are also encouraged to follow these protocols when entering Australia (Wildlife Health Australia, 2019). In Europe, the European Federation of Speleology (FSE European Working Group on WNS, 2010) recommends cavers should reduce the number of visits to bat hibernacula, clean equipment with soap water when changing caving areas, and decontaminate equipment when traveling across continents. However, the role of humans as *P. destructans* vectors is not obvious to the average cave visitor. Thus, the lack of published evidence from the field partly hinders the application of present restrictions and limits their conservation effectiveness.

The aim of the present study is to fill the current knowledge gap in preventative actions or behaviours, and to demonstrate cavers’ potential as *P. destructans* vectors. First, samples were collected from caving equipment used in underground sites to test for the presence and frequency of *P. destructans* spores on the equipment, and more importantly, spore viability. Second, lab experiments were conducted using deliberately contaminated pieces of caving equipment to trace *P. destructans* viability on the fabrics through time, while imitating typical storage conditions of such equipment. Lastly, an experimental test was performed to examine the effectiveness of common hygiene practices people most often apply while cleaning contaminated pieces of caving equipment in the absence of a *P. destructans*-decontamination protocol. The results demonstrate that human clothes/equipment can be a vector for viable *P. destructans* spores, though simple washing practices can significantly remove most spores. Finally, we advocate that simple preventive practices, if adopted at a large scale, by a large majority of cavers, would be more effective than stricter practices followed by few, and that education on pathogen pollution is greatly needed, irrespective of the presence or absence of an immediate crisis.

**MATERIALS AND METHODS**

### I. Isolating *P. destructans* from caving equipment

**Sample collection**

Swabs from caving equipment were collected during two consecutive field seasons (2017 and 2018) in Bulgaria. Sampling sites included two limestone caves, Ivanova voda (N41.89, E24.88) and Balabanova dupka (N43.13, E23.04), where *P. destructans* presence was previously confirmed by culture and genetic analysis both on bats and cave walls (Zhelyazkova & Puechmaille, unpublished data). In both caves, passages are present that require climbing and/or crawling. Thus, contact between clothes and cave substratum is inevitable, which is typical for caves that are not tourist destinations. During each visit, 5 samples were taken from researchers’ equipment (caving suits, gloves, boots, harnesses, and bags; previously washed with soap and water) before entering the cave to control for *P. destructans* contamination from previous caving trips. Five more samples were taken from the same pieces of equipment (and the same area of those pieces of equipment) upon exiting the cave (Supplemental Table). A sample consisted of approximately a 10 cm$^2$ area, swabbed using a dry sterile polyester swab (COPAN Diagnostics, Murrieta) that was then placed in an empty sterile Eppendorf tube and taken to the lab for cultivation. When swabbing a caving boot, the upper part of the boot was chosen to avoid over-collecting of mud (Fig. 1A). When swabbing a caving suit, the areas of the knees or the elbows were chosen as they are typical for caves that are not tourist destinations.

A total of 100 samples were collected from 10 trips (10 samples per trip, 5 on entry, 5 on exit). Three samples were lost in the field and did not reach the lab.
Cultures
To evaluate the presence of viable *P. destructans* spores in a sample, cultures were used instead of the classic DNA-based detection methods (e.g., qPCR). DNA-based detection methods are informative for the presence and quantity of *P. destructans* DNA in a sample but are uninformative concerning viability. Swabs were plated on DPYA (Dextrose Peptone Yeast extract Agar) growth medium prepared as described in Vanderwolf et al. (2016). The swab was dabbed 5 times onto the plate, then 100 µl dH₂O added, and spread with a sterile microbiological spatula. Dishes were sealed with parafilm and stored inverted in the dark at 4°C. Colonies showing a morphology similar to *P. destructans* were isolated and then genetically confirmed as *P. destructans* as they successfully amplified for a panel of 18 microsatellites and two mating-type markers (Drees et al., 2017a; Dool et al., 2020).

II. *P. destructans* spore viability on caving equipment
A schematic of the spore viability experiment is presented in Figure 2.

Material preparation
To quantify *P. destructans* spore viability on caving equipment, three of the most popular materials used by speleologists were tested: cordura fabric for caving suits (Yana, Sofia), PVC fabric for caving suits (Lanjoff, Sofia), and a 9 mm nylon rope (Beal, Sofia). The fabrics were cut into 2 cm² squares, and the rope into 2 cm long pieces. To create sterile conditions, all materials were autoclaved prior to the experiment, which did not visibly change their characteristics. Cave sediments from Svinskata dupka cave (N43.08 E23.38) were collected, autoclaved, and manually homogenized prior to the experiment. Sediments were sticky, containing more clay than sand. As *P. destructans* isolates may vary in their survival and viability patterns, a mix of cultures in a spore solution was used to investigate the general species characteristic. *P. destructans* spore solution was prepared using a mixture of three randomly selected cultures originating from bats in Ivanova voda (Gd_2219-baa, Gd_2225-aaa, Gd_2227-aaa; The isolates can be obtained from the corresponding author upon request), the identity of which had been previously confirmed by microsatellite genetic analysis as described above. Fungal material (including hyphae and spores) was scraped off the plate and mixed with dH₂O in a 2-ml Eppendorf tube and homogenized manually and using a vortex.
At the time of scraping, cultures were approximately 4 months old. Spore concentration was determined using a Bürker cell-counting chamber and the average of 10 counts was used. Finally, dilutions were made to achieve a concentration of $10^6$ spores/ml.

**Contamination procedure**

A total of 180 pieces of equipment (60 per material type) were used for the spore viability experiment. For cordura and PVC fabrics, approximately 50 mg of sediment were placed in the center of each piece and 100 µl of spore solution added on top. Sediment and spore solution were mixed manually for 30 seconds using a small metal spoon and distributed evenly over the whole surface of the fabric piece. For the rope, the same quantities of sediment and spore solution were used, but the sediment was wet prior to applying it to the rope. All procedures were performed under sterile conditions and spore solution was homogenized by vortexing prior to pipetting.

**Storage conditions**

All pieces of equipment were divided into 2 storage groups. In Group 1, pieces were left to dry for 5 hours after contamination (until they were visibly completely dry). They were stored in an illuminated space at room temperature (20 to 23°C) imitating equipment that was left to dry without washing. In Group 2, pieces of equipment were left wet after contamination. They were stored in a bag, in the dark, at the same temperature imitating equipment left in a backpack. To avoid contamination with airborne microbes, which would hamper the isolation of viable *P. destructans* spores, samples from both groups were placed in empty sterile Petri dishes sealed with parafilm. For Group 1, 9-cm dishes were used, and for Group 2, 6-cm dishes were used to keep humidity higher. Five pieces (=replicates) of equipment were placed in each dish. In the smaller dishes, different pieces were slightly touching each other.

**Spore retrieval and plating**

Five replicates (each randomly taken from a different storage dish) of each of the three materials from each storage group (dry & wet) were taken for spore retrieval every 5 days over a period of 25 days starting from day 0 of the experiment (Fig. 2). This resulted in 180 (3 materials * 5 replicates * 2 storage conditions * 6 storage periods) pieces of material. A single piece of contaminated equipment (each piece was used only once) was put in a sterile 50-ml tube filled with 10 ml of ultrapure water using sterile tweezers, and vortexed for 20 seconds. The resulting solution was plated on 9-cm Petri dishes with DPYA growth medium. Two different plating dilutions were used – the first one using 100 µl of the solution, and the second one using 500 µl of solution. Two dilutions were used as it was not possible to have precise predictions regarding the number of *P. destructans* spores potentially germinating over the course of the experiment. Dishes were sealed with parafilm and stored inverted in the dark at 4°C. Fungal colonies (CFU’s) were counted manually after 2 weeks.

**III. Cleaning effectiveness**

A schematic of the cleaning effectiveness experiment is presented in Figure 3. PVC fabric was excluded from this test as the previous spore viability experiment detailed above showed a very low survival rate of *P. destructans* on PVC in wet conditions (see Results section). Thus, it was assumed any washing treatment including water would completely kill the fungus on PVC. Pieces of cordura fabric and rope were prepared and covered with *P. destructans* contaminated sediment as described for Group 2 above (i.e., ‘wet’). Contaminated fabrics were stored overnight in empty sterile Petri dishes sealed with parafilm (to avoid airborne contamination) at room temperature and cleaned the following day. Five replicates per material were used for each cleaning treatment and five replicates that were contaminated but not cleaned served as controls. Autoclaved tap water was used for all treatments.

![Fig. 3. Schematic of the cleaning effectivness experiment with nylon rope as an example. The same procedure was performed for cordura fabric pieces. Equipment contamination, spore retrieval, and plating were performed as shown in Figure 2.](image)

**Handwashing**

A hygiene practice commonly used by cavers consists of brushing dirty equipment in a bathtub or a river (usually with cold water) to physically remove the most prominent mud from the materials. To imitate this under laboratory conditions, a sterile toothbrush was used to remove *P. destructans* contaminated sediment from each piece of the tested equipment. Five pieces were put together in a 9-cm Petri dish filled with water at room temperature. Each piece was brushed for 10 seconds, then rinsed with 1 ml of water.

More cautious cavers who are informed about the risk of pathogen pollution may also add soap to
the water before brushing their equipment, as this recommendation is part of a protocol by the European Federation of Speleology regarding the prevention of *P. destructans* movement within Europe (FSE European Working Group on WNS, 2010). To test this protocol, 5 pieces of contaminated equipment were placed in a Petri dish as described above. Each piece was first cleaned with a sterile toothbrush for 5 seconds, next with a brush dipped in common liquid soap (Palmolive, Sofia) for 10 seconds, and finally rinsed with 3 x 1 ml of water, which was needed to completely remove the soap.

‘Washing machine’

To save time, many cavers choose to wash their equipment in a washing machine after brushing off the mud from the dirtiest parts. Most often, only water is used but, in some cases, detergents are also added. To imitate this scenario, the pieces of contaminated equipment were first brushed for 5 seconds as described above. The washing machine effect was imitated by 50-ml tubes placed in a roller incubator (with constant temperature maintained). Five pieces of equipment were placed in each tube and filled with water: 40 ml for the tubes containing cordura fabric and 30 ml for tubes containing rope. Three different temperatures (30°C, 40°C, and 50°C) were tested and for each temperature treatment, three tubes were prepared: water only, water and common liquid detergent (Ariel, Sofia), and water and specialized detergent for mountain equipment (NST Wash, Sofia). A volume of 200 µl of detergent was added to 40 ml of water, which approximated detergent to water ratio recommended by manufacturers. Temperatures above 50°C were not tested because they have been tested in other studies (Shelley et al., 2013) and because washing of life-supporting equipment (such as ropes and harnesses) at high temperatures is not generally practiced. Only two detergent types were tested as the aim of the present experiment was not to test market products, but simply to test if the addition of commonly used detergents significantly improved removal of *P. destructans*. All test tubes were rollers in the roller incubator set at the particular temperature (30°C, 40°C, or 50°C) for 30 minutes, then water in the tubes was changed 4 times to imitate rinsing.

Spore retrieval and plating were performed immediately after all treatments as described in the previous section (Experiment II). Fungal colonies were counted manually after 2 weeks.

IV. Statistical analysis

Statistical analyses were performed in R Studio (version 3.6.2). General linear models with a negative binomial distribution were used. Candidate models included all combinations of the explanatory variables, with or without interaction as specified below depending on the dataset. For each dataset, the Akaike information criterion with a correction for small sample sizes (AICc) was used to compare the fit of models. Subsequently, multimodel inferences were used for model averaging, whereby the model-averaged confidence intervals (95% CI) of the coefficients were calculated based on the cumulative weights of all models including the term. Explanatory variables with 95% CI not encompassing zero were considered to have a meaningful impact on the response variable.

For the spore viability experiment, the number of colonies enumerated in the dishes (Pd spore count) was used as a response variable, and the number of days of storage on the fabrics (0, 5, 10, 15, 20, and 25), material contaminated (cordura, rope, or PVC), and storage conditions (wet or dry) as explanatory variables.

For the cleaning effectiveness experiment, the type of washing (control vs ‘washing machine’ and handwashing), and material contaminated were used as explanatory variables. For the ‘washing machine’ scenario, temperature (30, 40, and 50°C), detergent (water only vs specialized and standard detergent), and material contaminated were used as explanatory variables. For the handwashing part, detergent (brush only vs brush & soap), and material contaminated were used as explanatory variables. Again, the number of colonies enumerated in the dishes (Pd spore count) was used as a response variable. For both experiments, the count data from dishes where 100 µl of spore solution was plated were used as enumeration was more reliable due to less plate filling. Increases or decreases in germination of *P. destructans* spores for different treatments are presented as an incidence rate ratio (in comparison to the control group). Graphs were prepared with ggplot2 (Wickham, 2016).

I. Isolating *P. destructans* from caving equipment

A total of 12 *P. destructans* cultures were obtained (Table 1) from 9 of 25 samples collected after visits to Ivanova voda (Supplemental Table). At least 1 culture was obtained from each of the 5 field trips. From Balabanova dupka, a total of 12 *P. destructans* cultures were obtained (Table 1) from 5 of 24 samples (one sample was lost in the field) collected after visits to the cave (Supplemental Table). Out of 5 field trips, 3 returned samples positive for *P. destructans*. No *P. destructans* was isolated from any of the control samples collected before cave entry.

II. *P. destructans* viability on caving equipment

Storage time (from 0 to 25 days, tested every 5 days) of the caving equipment under room temperature did not lead to a significant reduction in *P. destructans* germination rate (Coefficient [95% CI]: 0.0129 [-0.0116 – 0.0376]; Fig. 4). It is important to highlight significant differences in overall viability depending on the material used. When using cordura fabric as the reference, the estimates of *P. destructans* spore recovery for rope and PVC fabric pieces were 52 and 69% smaller respectively (rope: -0.9100 [-1.3746 – -0.4455]; PVC: -1.3519 [-1.8650 – -0.8388]). Interestingly, in both dry and wet conditions, a peak in spore germination was observed on cordura fabric pieces after 10 to 15 days of storage. The interaction between storage condition (dry versus wet) and the material type was only significant for
the PVC fabric (-4.9564 [-6.8718 – -3.0411]): on dry PVC, *P. destructans* remained viable till the end of the experiment (25 days), but on wet PVC fabric, *P. destructans* did not survive after the first 5 days (Fig. 4).

**III. Effectiveness of cleaning**

When compared to the control group, a significant reduction in the number of *P. destructans* spores on the fabrics could be seen after both ‘washing machine’ treatment (decrease of 95%, -3.0169 [-4.2745 – -1.7592]) and handwashing (decrease of 94%, Coefficient [95% CI]: -2.7493 [-4.2215 – -1.2770]) (Fig. 5). In the ‘washing machine’ treatments, higher temperature improved cleaning effectiveness (40°C, 55% decrease in *P. destructans* spores compared to 30°C, -0.8055 [-1.2684 – -0.3425]; 50°C, 80% decrease in *P. destructans* spores compared to 30°C, -1.6085 [-2.1406 – -1.0764]). Adding a detergent, both standard and specialized also contributed to the reduction of germinating *P. destructans* spores by 97 and 82% respectively compared to the use of water only (standard: -3.5583 [-4.3665 – -2.7501]; specialized: -1.7023 [-2.1575 – -1.2470]). Material type was a significant factor, the rope being cleaned less successfully than the cordura fabric (256% increase in *P. destructans* germination, 1.2694 [0.8445 – 1.6942]). In handwashing, the treatment type (i.e., use of water or soap with the brush) was not significant (-0.4294 [-1.1474 – 0.2887]). As in the washing machine experiment, the rope was again cleaned less successfully compared to the cordura fabric (130% increase in *P. destructans* germination, 0.8197 [0.1081 – 1.5312]).

**Table 1.** Samples positive for the presence of *P. destructans*, collected from various caving equipment substrates after visiting Ivanova voda and Balabanova dupka caves in 2017 and 2018. Species identity of positive cultures was confirmed by microsatellite genetic analysis as described by Dool et al. (2020).

<table>
<thead>
<tr>
<th>Sample</th>
<th><em>P. destructans isolate</em></th>
<th>Site</th>
<th>Date</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>20170318_IVA_C_42</td>
<td>Gd_02169-aa</td>
<td>Ivanova voda</td>
<td>18/03/2017</td>
<td>cordura suit</td>
</tr>
<tr>
<td>20170409_GBL_C_62</td>
<td>Gd_02181-aa</td>
<td>Balabanova dupka</td>
<td>09/04/2017</td>
<td>caving bag</td>
</tr>
<tr>
<td>20170409_GBL_C_64</td>
<td>Gd_02182-aa</td>
<td>Balabanova dupka</td>
<td>09/04/2017</td>
<td>cordura suit, leg</td>
</tr>
<tr>
<td>20170609_GBL_C_61</td>
<td>Gd_02204-aa</td>
<td>Balabanova dupka</td>
<td>09/06/2017</td>
<td>PVC suit, leg</td>
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</table>
cavers’ equipment upon exiting underground sites, demonstrating that cavers can potentially spread *P. destructans* between sites.

**Importance of *P. destructans* viability on caving equipment**

Results from the lab experiment further showed that *P. destructans* spores can germinate after storage on dirty fabrics (cordura, nylon rope, and PVC) for at least 25 days at room temperature under both wet and dry conditions (except for wet PVC). This is ample time for human travel to occur across countries and continents and effectively spread the pathogen between geographically distant sites. *P. destructans* viability was not investigated for longer than 25 days and results obtained in the present experiment might vary depending on fungal isolates, cave sediment, brands of equipment, or storage conditions. However, the observed trends should be robust to such variations and the marginal decays in germination rates suggest that some spores might even remain viable for several months or longer on caving equipment. This is supported by another experiment (Fischer et al., 2020) with contaminated concrete blocks where *P. destructans* spores stayed viable for at least two years at 10°C, even in the absence of nutrients. Based on model estimates, Reynolds et al. (2015) speculate that *P. destructans* populations can survive in sediments from bat hibernacula for decades, and studies in other species indicate that fungal spores are among the most stress-resistant eukaryotic cells described to date (Wyatt et al., 2013). As reaching a suitable habitat seems to be the main factor for a successful alien species invasion (Skarpaas et al., 2009), caving equipment could be a particularly good *P. destructans* vector, being transported not between random sites but between caves (or mines) that often offer similar environmental conditions across the globe. In many countries, caving and cave tourism are becoming increasingly popular activities, supported by more affordable international travel, thus greater research and awareness on the subject are needed.

**Significance of materials**

Regarding the difference in *P. destructans* survival on tested materials, a detailed interpretation of the results requires further investigation. The peak in spore germination observed on cordura fabric after 10 to 15 days of storage is consistent with other *P. destructans* survival/viability experiments (Fischer et al., 2020). Many factors have been demonstrated to stimulate or inhibit fungal spore germination (e.g., Wyatt et al., 2013), however, there is not enough information to explain the difference in *P. destructans* germination curves on cordura fabric and nylon rope. The almost 100% mortality of *P. destructans* spores on wet PVC suit pieces could be explained by the proven toxicity of plasticized PVC leaking in water (Lithner et al., 2012). Whether this should be considered as a promise for *P. destructans*-killing caving equipment or as a warning for potential sources of underground water pollution, is a worthy question for further research.

**DISCUSSION**

**Cavers as effective vectors of *P. destructans***

Results of the present research clearly demonstrate that during human visitation to caves, clothing and equipment can become contaminated with viable *P. destructans* spores, irrespective of fabric types or time of the year (before or after bat hibernation). Despite the limited surface area swabbed and the relatively low number of samples collected per trip, viable *P. destructans* was cultured following almost every cave visit. Assuming that a caving suit is made of roughly 2 m² of fabric, 1–2 *P. destructans* spores isolated from an area of 10 cm² may indicate the potential for hundreds of spores to be present on a whole suit. Additionally, given that the cultivation method does not detect all viable material present in a sample, the true number of *P. destructans* spores present on caving clothes may be underestimated. The number of *P. destructans* spores on caving equipment is surely partly dependant on the contact rate between the equipment and the environment, which varies between sites, between cavers within a site and between areas of the same caving suit. Furthermore, the amount of *P. destructans* DNA drastically varies between and within sites as well as within seasons (Hoyt et al., 2020). For example, within sites, variations can be related to the type of sediments, proximity to bat colonies, and distance from the entrance or height from the floor (Verant et al., 2018). Thus, variation in the amount of viable material can also be expected. Irrespective of these variations which are ubiquitous under field conditions and beyond the scope of the present study, viable *P. destructans* was isolated from...
Broader relevance to pathogen pollution in caves

The overall conclusion that humans act as vectors of *P. destructans* spores is likely valid for many if not most species of cave fungi. These include bat specific pathogens such as *Trichophyton reedelli*, currently not associated with mortality events but still poorly investigated with reports only from North America (Lorch et al., 2015), or human specific pathogens such as *Histoplasma capsulatum*, the most frequent cause of fungal respiratory infection with a potentially lethal outcome (Deepe, 2015). Over 1,000 described species of fungi, slime moulds, and yeasts have been documented in caves and mines worldwide (e.g., Vanderwolf et al., 2013) but total species count is expected to be much higher. For example, based on sampling at only four caves and two mines from New York, Zhang et al. (2014) identified 248 Operational Taxonomic Units of fungi, however the species accumulation curve showed no sign of reaching a plateau. Hence, the number of fungal species per site is likely in the hundreds, if not thousands, and all these may be capable of adhering to caving clothing and equipment, especially when coated in cave sediment. Therefore, the potential risk of spreading cave microbes, regardless of known pathogenicity, should be carefully considered by all visitors to underground sites anywhere on the globe.

Disinfection procedures and hygiene

Not surprisingly, the experiments from the current study revealed that hygiene is an important factor for limiting *P. destructans* load on caving equipment and thus reduces the risk of pathogen pollution in caves. They complement the results obtained by Shelley et al. (2013) who tested multiple detergents and exposure temperatures that could kill *P. destructans* on fabrics. The most appropriate hygienic technique recommended by Shelly et al. (2013) was immersion in water above 50°C for at least 25 minutes and/or applying a list of commercially available chemicals, which demonstrated an effective kill rate greater than 99.995%. The aim of the present study was not to improve this rate, but to examine regular hygiene practices used by cavers in Europe and to test whether such practices approach decontamination effectiveness. In accordance to Shelley et al., the present results showed that simply brushing off the mud from caving clothes and equipment greatly reduced the amount of *P. destructans* spores on the fabrics with an average of 95.3 of spores being removed from the cordura fabric pieces and an average of 85.1% being removed from the nylon rope. Increased time of washing, adding detergents, and/or raising the temperature further improved cleaning effectiveness. The best results were obtained by washing with standard detergent at 50°C, which led to an average of 99.8% of *P. destructans* spores being removed from the cordura fabric pieces and an average of 98.7% being removed from the nylon rope. The difference between tested materials may be due to the porous texture of the rope, which would hamper the physical removal of contaminated mud particles. It is also important to note that while washing with standard laundry detergents at a temperature of 50°C or above is appropriate for caving suits, this is not true for life-supporting equipment such as ropes and harnesses. For ropes and harnesses, manufacturers usually recommend washing at temperatures up to 30°C either with no detergent or with specialized detergent only. Caving rope is not typically washed using a washing machine and specialized equipment detergents are often cost prohibitive, thus it is realistic to assume that ropes and harnesses are only being washed with water at room temperature, and this treatment showed the lowest relative effectiveness in *P. destructans* removal in the present study.

Practical advice for cavers’ behaviour

Given the evidence of potential transport of *P. destructans* and/or other species of cave microbes by cavers via clothes and equipment, we recommend that hygiene practices (that are neither difficult nor expensive) to clean clothes and equipment occur after every visit to an underground site at any point on the globe. Although this will not completely prevent pathogen pollution in caves or elsewhere, it can drastically reduce propagule pressure, measured as the number of alien individuals transported and the number of introduction events – overall reducing the chance of successful establishment and invasive population success (Lockwood et al., 2005). However, it is important to emphasize that reducing *P. destructans* load on caving equipment does not necessarily eliminate it, and that hygiene is not a substitute for decontamination. Therefore, existing decontamination protocols are still necessary when visiting sites of significant risk (e.g., sites in the USA and Canada) or when traveling to remote sites where the consequences of bringing alien microflora are not known (Zhelyazkova et al., 2019). Regarding life-supporting caving equipment that cannot be properly decontaminated, site-dedication (or region-dedication) would be the most appropriate option, especially for long distance and international travel.

Future perspectives

As the rate of biological invasions worldwide continues to increase (Hulme, 2009) and the consequences of such invasions are sometimes seen a century later (Essl et al., 2011), one cannot be too cautious when tackling the subject. To manage pathogen pollution (including but not limited to *P. destructans* and WND) in caves and elsewhere, a combination of both reactive (e.g., following specific decontamination protocols and locally adapted recommendations) and preventive (e.g., good hygiene) strategies should be considered. Of particular importance is communication with the target public as any measures, reactive or preventive, can only be effective if they are understood and accepted by the majority of communities. As most of the caving clubs accept new members after they complete a specialized course, it would be practical if pathogen pollution prevention could be included in the curriculum of such programs. Practicing hygienic techniques on equipment should be a normalized behaviour after every cave visit and reviewing potential
risks for human and environmental health should become an obligatory part of expedition preparation. Additionally, it is important to distribute existing cleaning/decontamination protocols outside their countries of origin, evaluate the degree of awareness on the subject among people involved in caving activities, and assess the distribution and application of such recommendations within the caving community. We are convinced that conservation questions such as pathogen pollution prevention should go further than localized academic pursuits and become a mutual responsibility of our society in this time of globalization.

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Authorship statement: VZ and SJP conceived the study. VZ and SJP designed the study with input from AH, GR and NT. VZ, AH, and NT organised fieldwork and collected the samples. VZ performed the experiments. GR provided lab space, consumables and technical assistance. VZ and SJP analyzed the data and wrote the paper with input from all authors.

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The role of microhabitats in structuring cave invertebrate communities in Guatemala

Gabrielle S.M. Pacheco, Marconi Souza Silva, Enio Cano, and Rodrigo L. Ferreira

Abstract: Several studies have tried to elucidate the main environmental features driving invertebrate community structure in cave environments. They found that many factors influence the community structure, but rarely focused on how substrate types and heterogeneity might shape these communities. Therefore, the objective of this study was to assess which substrate features and whether or not substrate heterogeneity determines the invertebrate community structure (species richness and composition) in a set of limestone caves in Guatemala. We hypothesized that the troglobitic fauna responds differently to habitat structure regarding species richness and composition than non-troglobitic fauna because they are more specialized to live in subterranean habitats. Using 30 m² transects, the invertebrate fauna was collected and the substrate features were measured. The results showed that community responded to the presence of guano, cobbles, boulders, and substrate heterogeneity. The positive relationship between non-troglobitic species composition with the presence of guano reinforces the importance of food resources for structuring invertebrate cave communities in Guatemalan caves. Furthermore, the troglobitic species responded to different substrate features when compared to non-troglobitic species. For them, instead of the presence of organic matter, a higher variety of abiotic microhabitats seem to be the main driver for species diversity within a cave. The high specialization level of troglobitic organisms might be the reason why they respond differently to environmental conditions. The findings of this study highlight the importance of biological surveys for understanding cave biodiversity and give insights on how this biodiversity might be distributed within a cave. Conservation measures should keep in mind the target organisms and if such measures aim to protect a broad variety of organisms, then one should aim to preserve as many microhabitats and trophic resources as possible.

Keywords: arthropods, habitat heterogeneity, substrate composition, troglobites, Central America

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INTRODUCTION

Patterns of species distribution and factors structuring communities have always been central subjects for ecological studies (Dunson & Travis, 1991; Kolasa & Pickett, 1991; Cushman & McGarigal, 2004; Steinitz et al., 2006; Talley, 2007). For caves, understanding these patterns and factors is fundamental for conservation purposes, as the organisms that inhabit these environments are known to be relatively sensitive to many stressors, both natural and anthropic (Howarth, 1983; Mammola, 2019; Mammola et al., 2019).

Several studies have tried to elucidate the main environmental factors driving the invertebrate community structure (species richness and composition) in cave environments. The main factors highlighted as important are seasonality (Tobin et al., 2013; Mammola et al., 2015; Bento et al., 2016; Lunghi et al., 2017; Kozel et al., 2019), lithology (Souza-Silva et al., 2011b), landscape structure (Christman et al., 2016; Pellegrini et al., 2016; Mammola & Leroy, 2017), distance from cave entrance (Ferreira & Martins, 1998; Prous et al., 2004; Tobin et al., 2013; Kozel et al., 2019), linear development of the cave (Simões et al., 2015; Pellegrini & Ferreira, 2016), presence
of water (Simões et al., 2015), resource availability (Ferreira & Martins, 1998; Gers, 1998; Jaffé et al., 2016; Ferreira, 2019), microclimate (Mammola et al., 2015; Lunghi et al., 2019) and speleogenesis (Sendra et al., 2014; Jiménez-Valverde et al., 2017). However, rare examples focused on how the substrate types and substrate heterogeneity might be shaping these communities within a cave (Prous et al., 2015; Pellegrini et al., 2016; Zepon & Bichuette, 2017).

It is currently known that invertebrates have a preferential selection of microhabitats inside the caves (Culver & Pipan, 2009; Moseley, 2009; Souza-Silva & Ferreira, 2009; Mammola et al., 2016). Such preferences for specific types of microhabitats are due to a variety of reasons, including behavioral, physiological, and morphological adaptations (Howarth, 1983; Zepon & Bichuette, 2017). These microhabitats encompass different abiotic (lakes, water bodies, puddles, types of substrates, and rocks of different textures and sizes) and biotic (roots, guano, vegetal debris, and carcasses) features (Ferreira et al., 2007; Culver & Pipan, 2009; Souza-Silva et al., 2011a; Simões et al., 2015). Therefore, the habitat heterogeneity increases the microhabitat availability for the invertebrate fauna (Zagmajster et al., 2018).

Organisms that are specialized to live in caves (i.e., troglobites) are, in many cases, rare and endemic. They are vulnerable to stochastic events, environmental disturbances, and anthropic impacts (Gibert & Deharveng, 2002; Culver & Pipan, 2009, Mammola et al., 2019). Furthermore, underground life provides environmental ecosystem services, such as the decomposition of organic matter, pollination, and insect control, features that have been attracting the attention of conservation researchers (Culver & Pipan, 2009, Mammola et al., 2019).

Guatemala has a variety of caves in quite distinct regions, although in a small territory. There is a reasonable amount of scientific work on the taxonomy of cave invertebrates in the country (see Reddell, 1981; Strinati, 1994; Reddell & Veni, 1996), but there is a noticeable gap when it comes to their ecology. This study, therefore, aimed to evaluate which substrate features influence the invertebrate community structure and how the substrate heterogeneity influences the invertebrate community structure in a set of Guatemalan caves. We hypothesized that places with higher substrate heterogeneity have higher species richness than places with lower substrate heterogeneity and that the troglobitic species respond differently to the habitat structure than non-troglobitic species because they are more specialized to live in subterranean habitats.

MATERIALS AND METHODS

Study area

Guatemala is a tropical country that features several environmental conditions within a relatively small geographic area (Strinati, 1994). With three different climate types (Equatorial Monsoon, Equatorial savannah with dry winter and Warm temperate climate with dry winter) (Kottek et al., 2006; Peel et al., 2007), the country features an average of 3,000 to 4,000 mm of rain every year, with rains concentrated between May and October (Kottek et al., 2006; Peel et al., 2007). There is a set of limestone karstic landscapes that cover about one-third of its area. The karstic landscapes from Guatemala are divided into four main geologic domains, mostly in the northern portion of the country: Huehuetenango, Alta Verapaz, Izabal, and Petén (Strinati, 1994). This study was conducted in 10 limestone caves from two of these karstic regions: Alta Verapaz and Petén, on a region known as Northern Lowlands (Fig. 1; Table 1).

The caves sampled had different characteristics. They varied as to the presence/absence of water bodies, dripping activity, and the number of entrances. The caves also had different uses. Furthermore, most of the caves had remains of Mayan rituals, such as clay pots, candles, bones, and traces of bonfires.

Cueva Aktun Kan, Cúpula de los Mucílagaos, Ventanas de Seguridad, Cueva Blanca, and Gruta de Lanqun have different levels of touristic activity. Cueva Aktun Kan and Gruta de Lanqun are located in places with high international tourism influx and are, therefore, highly explored touristically with almost no control whatsoever. The other touristic caves, in the other hand, are located in an area predominantly visited by local tourists and have more controlled adventure tourism. Cueva del Venado, Cueva el Rostro, Cueva de Arcilla, Cueva Coral, and Cueva Chipix do not have touristic activity.

Field procedures

All data were collected in 30 m² (10 m x 3 m) transects throughout the caves. All transects were placed at least 25 m from each other. The number of transects per cave ranged from one to eight (Table 1), with a total of 25 transects. In this study, the number of transects per cave was not standardized for logistical reasons, therefore it was best suited to use the transects as sampling units instead of the caves. Since there are no studies that state how far apart cave sampling units should be in order to assume independency, we have assumed a 25 m distance between transects as a measure of independence among transects, especially considering the highly heterogeneous substrates sampled within each cave.

The invertebrate fauna was collected manually in the transects through direct intuitive searches (Wynne et al., 2019) and the sampling method was exhaustive (i.e., the researchers only stopped once there were no more invertebrates to be collected or accounted for). The invertebrates were collected with the aid of brushes and tweezers and placed in vial containing a solution of 70% ethanol for further examination and identification in the laboratory. When the abundance of invertebrates of the same species in a transect was high, a few specimens were collected and the remaining individuals were accounted for in the field notebook.

Afterwards, the substrate was characterized according to a field protocol adapted from Peck et al. (2006) and Hughes and Peck (2008). This protocol consists of dividing each transect into 10 sections of...
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one meter each and estimating the percentage of each substrate type. In this case: guano, roots, leaves, twigs, trunks, waterbody, water puddle, actinomycetes, fine sediment (0.06 – 64 mm), cobbles (64 – 1000 mm), boulders (1000 – 4000 mm), matrix rock (>4000 mm) and human interventions (bridges, pathways, and steps made of concrete).

Because we have chosen a visual estimation method for evaluating the substrate percentages, three steps were taken in order to minimize observer error: (i) all transects were characterized by the same person; (ii) the percentage values obtained in the 10 sections in a transect summed and divided by 10 (arithmetic mean), resulting in a single percentage value for each substrate type in the transect and (iii) these percentage ranges allocated into five categories:

\[<1\% = 0; 1 – 5\% = 1; 5 – 25\% = 2; 25 – 50\% = 3; 50 – 75\% = 4; 75 – 100\% = 5.\]

Species identification and troglomorphisms

Invertebrates were identified until the lowest possible taxonomic level with the aid of taxonomic keys and then grouped into morphospecies (Oliver & Beattie 1996). This approach was chosen due to a large number of new species found in caves, combined with the lack of literature and specialists on some of the taxa. Still, specimens of the taxa Acari, Diplura, Isopoda, Opiliones, Palpigradi, and Pseudoscorpiones were identified by specialists.

Specimens that featured morphologic traits that indicate adaptations to life in the cave environment were considered to be potentially troglobitic. These
characteristics classically include the elongation of locomotor and sensory appendages, increased number of trichobothria, absence or reduction of ocular structures, body pigmentation, and wings (Barr, 1968; Hoch & Ferreira, 2012; Novak et al., 2012).

**Data analysis**

To test the influence of the percentage of each substrate type (guano, roots, leaves, twigs, trunks, waterbody, water puddle, actinomycetes, fine sediment, cobbles, boulders, matrix rock, human interventions) and substrate heterogeneity on species composition and richness, Distance-Based Linear Models (DistLM) were performed in Primer-e and Permanova + Software (Anderson et al., 2008). The model selection was made using the R² selection criterion and forward selection procedure. These models had the transects as sample units and only significant predictor variables were included in the final best models. To observe the effects on species composition, we built the resemblance matrix using the Bray Curtis similarity index with the invertebrate data transformed into the squared root, in order to mitigate the effect of large species abundances and to observe the effects on species richness, we built models using an Euclidean Distance matrix.

To test our main hypotheses, the above-cited analyses were performed for non-troglobitic species and then repeated using only troglobitic species. Significance was regarded at p ≤ 0.05.

**RESULTS**

A total of 10,354 specimens were registered in the 25 sampled transects. They were distributed into 38 orders, at least 78 families and 177 species, 24 of which were considered troglobites (Fig. 2 and Supplementary Material). The troglobitic fauna includes one species of Araneae (Corinnidae), one Coleoptera (Tenebrionidae), two Diplura (Campodeidae), five Collembola (Entomobryidae and Symphypleona), one Geophilomorpha, two Isopoda (Philosciidae and Styloniscidae), two Zygentoma (Nicoletiidae), two Opiliones (Samoidae and Stygnopsidae), four Polydesmida, and four Pseudoscorpiones (one Bochicidae and three Chthoniidae). The highest non-troglobitic species richness was found in the transects of Gruta de Lanquin (29 spp.), Cueva Chipix (29 spp.), and Cueva Aktun Kan (28 spp.), while the highest troglobitic species richness was found in the transects of Gruta de Lanquin (6 spp.), Cueva Blanca (4 spp.), and Cueva Aktun Kan (4 spp.).

Fig. 2. Some of the troglobitic species sampled in Guatemala caves. a) Araneae (Corinnidae); b) Diplura (Campodeidae, Juxtlacampa sp. n); c) Isopoda (Styloniscidae); d) Pseudoscorpiones (Ideorontidae, Typhloroncus guatemalensis); e) Opiliones (Stygnopsidae); f) Thysanura (Nicoletiidae, Anelpistina sp. n); g) Polydesmida (Chelodesmidae); h) Polydesmida (Pyrgodesmidae); i) Polydesmida (Paradoxosomatidae).
The most abundant substrate type was fine sediment, followed by matrix rock, guano, cobbles, and boulders, present in 24, 20, 16, 14, and 11 of the 25 transects, respectively (Supplementary Material). Each transect had an average of 4.19 (sd = ±1.48) different types of substrates. The transects with higher substrate heterogeneity were located in Cueva Chipix (transect 10, H’ = 2.008), Cueva Coral (Transect 9, H’ = 1.864), and Gruta de Lanquín (transects 1 and 4, H’ = 1.748) (Supplementary Material).

The non-troglobitic species composition variation was best explained by the presence of guano, cobbles, and substrate heterogeneity, which explained together 23.1% of the variation on the community on the sequential tests and 27.9% of the non-troglobitic species richness variation was best explained by the substrate heterogeneity (Table 2). As for the troglobitic species, the variation in species composition was best explained in the model by the presence of boulders, substrate heterogeneity, and cobbles, totaling 24.3% of explanation power on the model, while 20% of the troglobitic species richness variation was best explained by the presence of boulders (Table 2).

<table>
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<th>Species composition</th>
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<th>Cobble</th>
<th>Boulder</th>
<th>Subs heter</th>
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**DISCUSSION**

The species richness and composition found in this study are unprecedented for Guatemala. Previous studies focused mostly on descriptions of new species and thus failed to showcase the broad diversity of invertebrate species in Guatemalan caves (Barr, 1973; Gertsch, 1973; Schultz, 1977; Platnick & Pass, 1982; Rodriguez & Hobbs Jr, 1990; Espinasa & Zhuang, 2009; Viana & Ferreira, 2019) with rare exceptions (Mitchell & Reddell, 1973; Reddell, 1981; Reddell & Veni, 1996).

The linear models showed that the troglobitic species composition is more influenced by the substrate heterogeneity than the non-troglobitic, but only non-troglobitic species richness is influenced by substrate heterogeneity. Thus, for troglobitic species, the substrate heterogeneity might not be providing suitable habitats to a larger number of species, but instead for a larger number of individuals. This is probably a result of the high specialization of troglobitic organisms. While for the non-troglobitic fauna, the variety of substrates provides suitable habitats for an important increase in both species richness and composition.

In our study, non-troglobitic species behaved as already showed in the literature, as it is known that heterogenous habitats can shelter a larger number of species by providing more available habitats, allowing a decrease in ecological niche overlap and reducing competitive exclusion (Poulsen & Culver, 1969; Ferreira & Souza-Silva, 2001; Tews et al., 2004; Stein et al., 2014; Pellegrini et al., 2016; Resende & Bichuette, 2016). Environments formed by the agglomeration of gravel, for example, comprise void spaces that can provide terrestrial invertebrate species with microhabitats, food resources, protection, and refuge (Mammola et al., 2016).

Since most caves can be characterized as oligotrophic environments when compared to the surface environments (Fiser, 2019; Fong, 2019; Trontelj, 2019), the local presence of organic resources often means that these places have a higher diversity of invertebrate fauna. Several authors have already shown the importance of guano deposits and vegetal debris inside the cave environment (Decu, 1986; Gnaspini, 1989; Ferreira & Martins, 1998; Ferreira & Martins, 1999; Ferreira et al., 2000; Ferreira et al., 2007; Santana et al., 2010; Schneider et al., 2011). Beyond providing habitat availability, they also act as the main food sources for the lower levels of the food web, allowing the occurrence of greater species richness (Pellegrini & Ferreira, 2012; Pellegrini & Ferreira, 2013; Jaffe et al., 2016; Ferreira, 2019). The direct and significant relationship between non-troglobitic species composition with the presence of guano reinforces the importance of food resources for the structuring of Guatemalan cave invertebrate communities.
The presence of guano tends to be of extreme importance in permanently dry caves (Ferreira et al., 2007; Ferreira, 2019). In this study, even though most of the caves had water bodies, the guano was still a strong structurer for the cave invertebrate communities. Here, the presence of guano seems to be more important for the invertebrates than the presence of other types of organic matter (actinomycetes and vegetal debris). The abundance of guano in a cave can eventually attract a huge variety of invertebrates, most of them non-troglobites, which can lead to the emergence and establishment of large invertebrate populations and consequently a high level of biological interactions onto it. The presence of these communities on an ephemeral resource such as guano might repel the troglobitic species, which are often more sensitive, from these habitats (Ferreira et al., 2007), although there are exceptions (Ferreira, 2019).

Accordingly, the troglobitic species seem to be influenced differently from the non-troglobitic within the caves, responding to different substrate features when compared to non-troglobitic species. For them, the presence of organic matter, regardless of its source, does not seem to be the main driver for species diversity within a cave. Instead, troglobitic species composition seems to be more related to a higher variety of abiotic microhabitats (cobbles, boulders, and substrate heterogeneity) and troglobitic species richness, to the presence of boulders. The high specialization level of troglobitic organisms might be the reason why they respond differently to environmental conditions, in this case, substrate characteristics and heterogeneity. The lack of a statistically significant relationship between troglobitic species richness and substrate heterogeneity is a sign of such specialization level since instead of filling all available ecological niches and habitats, they seek for specific conditions inside the cave environment. In other words, substrate heterogeneity can influence which troglobitic species are inhabiting a cave but not how many. Troglobitic organisms might have suffered different ecological pressures over time when compared to troglophiles and trogloxenes, hence occupying an entirely different niche. Being adapted to the dark and oligotrophic cave environment, they can inhabit places that are not tolerable for most cave dwellers (Kozel et al., 2019).

The relatively low explanatory values obtained in the linear models were expected due to the methodology chosen for this study. There is likely a range of other parameters influencing these communities. Therefore, future studies should incorporate as many variables as possible to further investigate and elucidate the main factors influencing cave invertebrate fauna.

The findings of this study highlight the importance of biological surveys for understanding cave biodiversity and give insights on how this biodiversity might be distributed within a cave. Conservation biologists should keep in mind to target as many microhabitats as possible to maximize the effectiveness of protection measures. Furthermore, it provides a scientific biological background for the creation of new parameters for the management and conservation of caves in Guatemala.

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Authorship statement: RLF, MSS, and GSMP designed the research project; EC provided all infrastructure needed for the fieldwork in Guatemala; GSMP conducted all laboratory work, data analysis, and wrote the first manuscript draft; RLF, MSS, and EC revised the first draft, and helped to build the final manuscript.

REFERENCES


The importance of microhabitats for cave invertebrate communities.

International Journal of Speleology, 49 (2), 161-169. Tampa, FL (USA) May 2020


Caves and karst of the Congo Republic, Central, and Northern Gabon is a valuable book providing useful information for planning scientific and cave explorations in African karst areas. The author collected a large number of references throughout the speleological literature and the information reported in this book could be considered a kind of Baedeker (i.e., travel guide) concerning the caves of these areas. For this reason, in this review, the cave names are reported to provide more information to speleologist directly interested to caving in this African region. An overview of the cave exploration from the 1950s to the 2000s is reported. The geology and archaeology of the region are described providing fundamental details. With reference to the Congo Republic, in the area of the Niari Basin and Hangou Massif, the locations of the following caves are reported: Grotte de Diboueta, Grotte de Camp CCAF, Grotte Kounanga, Grotte de N'Zimou, Grotte de Micogny or M'Congny, and Grotte de M'Bouca. Reference of papers concerning some other caves are also reported. A map of an area between Loudima and Kimongo in the Niari Basin gives the location of Grotte de Doumboula, Grotte N’tari-Moundele, Grotte de Ferme Richard, and Grotte du Viaduc. In addition, papers concerning caves close to the confluence of the Niaru and Loubetsi rivers are listed. A short description of a few of them in this area, namely No. 1, No. 13 (Buffalo Tunnel), No. 14, No. 52, No. 61, No. 63, and No. 65 were published in 1961 in Annales de Spéléologie, volume 16, issue 2, p. 157-174.

In the area between Loudima and Kimongo, data on the following caves are listed: Grotte de Loudima, Grottes de Mallembé, Grotte du Viaduc, Grotte de Ferme Richard, Grotte N’tari Moundélé, Grotte de Doumbonia, and Grotte Kississi. In the mid-Niari Valley (north of the river) a map with the location of the following caves is reported: Grotte de Kila-N’tari, Grotte de Baukoussou, Aven de effondrement Ntadi Ndina, Grotte de Boas, Grotte de Kioudi, Perte de Mbumba, Resurgence de la Moumba, Grotte di Kicongo Grotte de Kikounou, Grotte de Ntadi Ba Ntie Ntie, and Perte de Ngouete. Data are also reported for the following caves: Ntadi Oubouka, Grotte de Bankoussou, Grotte de la Louati, Trou du Gendarme, Grotte de Kila-N’tari, Grotte de Kila-N’tari (with cave map), and Resurgence of the Kila, Grotte Boas. For the same valley (north of the river), other caves are listed with some description: Grotte L’Allemagne, Grotte de Ntadi Ba Ntie Ntie, Aven de effondrement Ntadi Ndine, Grottes de Kiéni, Roche Percée, Grotte de Ngaba Ngolo, Grotte de Kikongo, Grotte Kikounou, Ntadi Yomba, Ntadi Ku Maluende I-III, Perte de Mbumba, Resurgence de Mbumba, and Perte de Ngouete.

In the region around Bouansa, the following rock shelters are described: Ntadi Kimwanga, Ntadi Hele, Ha Mambwana Ma lutete, Ntadi Bi Gnumba, and Ntadi Hungu, whereas in the mining region of the Niari Valley, only two caves are cited: Grotte Galerie and Une Grotte à double issue.

Many limestone caves in the Massif of Foret de Bangou (a map containing their locations) are reported: Grottes de Matouridi, Grotte Mpassa, Grottes de la Lougoulou, Grotte Yengo, Grottes Missouril & II, Grotte de Mouounds, Grottes Meya I, II & III, Meya Village, Meya III, Grotte de Nzaa – Grotte M.Zac, Grotte Bernard, Unnamed Cave, Grotte de Ngabe Nzoke, Grotte Bittori, Grotte Laboratoire de Bittori, Grotte de Meya Nzoaari (with cave map), Grotte Paul, Grottes de M’Poka I & II, Grotte de Malala I-II, Grottes Bimb, Grotted Gilbert, Grotte Pierre, Grotte Rigobert, Grottes de Mousssa, and Grotte Kimankan.

The following caves are quoted in reference to bats colonies: Grotte “Apollinaire”, Grotte Bitiba, Grotte Bouaboumpono, Grotte Founta, Grotte Malonga Marc, Grotte Maféini, Grotte Mazabata, Grotte Mbangou, Grotte Missarvi, and Grotte Moutembessa. An area with possible karst phenomena is mentioned north of Brazzaville.
In Gabon, both coastal and central areas are considered. The following caves are recorded: *Grotte de Mabagna*, *Grotte de Boloukouboé*, *Grotte de Ngoun*, and *N'Toum Quarry*. In the area north of Fernan Viaz-Kamba Plain & Olandé River the following caves are documented: *Grottes de Mugumbi/Réseau de Mugumbi, Grotte des Minioptéres, Grotte Dinguembou*, and *Réseau de Lembé*.

In the Paleoproterozoic area of Central Gabon, a map showing the caves around Lastoursville is included along with the following caves: *Grotte Lihouma, Grotte de N'Doumbou or de Lastoursville (cave map), Grotte de Missié Grotte de Tao Tao, Grotte de Koubou, Grotte de Siyou 1 and 2, Grotte de Pahou, Grotte de Pahou 2, Grotte Youmbidi, Grotte de Mabelo, Grotte de Boukama*, and *Grotte de Ngongourouma*.

In conclusion, *Caves and karst of the Congo Republic, Central and Northern Gabon* is an excellent reference guide to any speleologist and/or cave explorer interested in studying and exploring the region.

**Arrigo A. Cigna**
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