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Survey of *Histoplasma capsulatum* in bat guano and status of histoplasmosis in Slovenia, Central Europe

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**Abstract:** There have been increasing reports on the presence of *Histoplasma capsulatum* in some European countries. The study investigated the presence of *Histoplasma* in bat guanos, speleologists with records of visiting *Histoplasma*-endemic regions and patients with histoplasmosis. A commercial ALPHA *Histoplasma* Antigen enzyme immunoassay was tested as an alternative methodology to detect *Histoplasma* in environment and compared with polymerase chain reaction (PCR) assays. The presence of *Histoplasma* antigen in bat guanos was not confirmed by PCR. Among 14 healthy speleologists, two were indicated as having the *Histoplasma* antigen in urine, but expressed negative PCR-specific results for the *Histoplasma* antigen. Five unequivocal cases of imported acute pulmonary histoplasmosis in Slovenia between years 2005 and 2016 were confirmed in patients returning from North and South America after visiting hazardous localities e.g., caves with guano, and places with dust. Currently there is no evidence of autochthonous histoplasmosis in Slovenia, or that bat guano is a source of *H. capsulatum*. Involvement of histoplasmosis in travellers’ and cavers’ morbidity might be underestimated in non-endemic areas. It is crucial to ensure the use of appropriate protective equipment in *Histoplasma* hazardous localities, to spread information about this hazardous microbe to vulnerable populations and to monitor the health of the environment. A differential diagnosis for a febrile respiratory disease outbreak in patients returning from endemic regions should trigger routine consideration of possible histoplasmosis.

**Keywords:** histoplasmosis, bat guano, caves, *Histoplasma* antigen EIA, speleologists

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**INTRODUCTION**

Histoplasmosis is an endemic mycosis caused by a dimorphic fungus with two distinct varieties that are pathogenic to humans, i.e., *Histoplasma capsulatum* var. *capsulatum* and *Histoplasma capsulatum* var. *duboisii*. The latter is known to be restricted to sub-Saharan Africa, whereas the former is distributed worldwide. *H. capsulatum* occurs in the soils of endemic areas, especially those contaminated by bird and bat droppings. Environmental disruption of *Histoplasma* habitats that introduces spores into the air is commonly a key factor associated with histoplasmosis outbreaks. Cave visitation, mining, building-site work, and agricultural activities are all associated with an increased risk of histoplasmosis (Kasuga et al., 2003; Kauffman; 2007 Antinori, 2014).

After inhalation of airborne *Histoplasma* microconidia, a majority of those exposed develop subclinical, self-limited, and commonly unrecognized disease symptoms. These are more likely to manifest after high-inoculum exposures, possibly with more intrinsically virulent strains, especially in immunocompromised patients and in very young and very old individuals. Those immunocompetent patients that do develop symptoms, most commonly present with acute pulmonary histoplasmosis, a »flu-like« illness, whereas severe or disseminated forms of histoplasmosis usually occur in immunocompromised individuals (Wheat et al., 2016).

Most reported cases of histoplasmosis in Europe are in immigrants or travellers returning from endemic areas, including the Americas, Africa, Southeast Asia, and East Australia (Bahr et al., 2015). According to an
epidemiological survey of the European Confederation of Medical Mycology Working Group, some parts of Italy might be considered endemic for \textit{H. capsulatum} (Ashbee et al., 2008). \textit{H. capsulatum} var. \textit{capsulatum} has been isolated from soil and animals in the Emilia Romagna region of Italy, and histoplasmin reactivity surveys in Lombardy, Tuscany and Apulia showed a low positivity in the general population (Sotgiu et al., 1966; Confalonieri et al., 1994; Mavropoulou et al., 2010; Reginato et al., 2014). In particular, the identification of some autochthonous cases in the Po Valley suggests the possible endemic presence of histoplasmosis in Italy (Manfredi et al., 1994; Farina et al., 2000, 2005; Calza et al., 2003; Ashbee et al., 2008). A few apparently autochthonous cases have been reported in other European countries, especially those in Southern Europe and those with badger populations (Ashbee et al., 2008; Craven 2013; Eisenberg et al., 2013), suggesting that extending the documentation of further case reports of histoplasmosis in Europe would give a completely different local picture of \textit{Histoplasma} epidemiology.

There are several reports of point-source outbreaks of acute pulmonary histoplasmosis among speleologists, so called «cave disease», largely but not exclusively related to cave exploration in the tropics and subtropics. The documented areas of cave disease in Europe are confined to underground spaces in Cyprus and to Topolniţa Cave in Romania (Craven, 2013). The diagnosis of histoplasmosis is indicated initially by the history of simultaneous exposure to bat guano and subsequent simultaneous onset of symptoms, and can then be confirmed in the individual by the presence of radiographic abnormalities and by the results of a suite of mycological and/or non-culture based tests. For such a potentially vulnerable population, it is of great importance to collect information about the prevalence of \textit{Histoplasma} in any particular underground system. The isolation of \textit{H. capsulatum} from environmental samples is difficult. The pathogen is typically retrieved as a cultivable strain after inoculation of laboratory animals (Richardson & Warnock, 2003). Direct identification of the fungus from complex environmental samples by use of molecular techniques is an alternative to the time-consuming culturing methods (Frias De Leon et al., 2012).

In this study we tested the usefulness of the commercial ALPHA \textit{Histoplasma} Antigen EIA kit intended for human in vitro diagnostics of histoplasmosis (Cloud et al., 2007) as a rapid and cost-effective alternative to culturing methods of inoculated laboratory animals for environmental samples. Since there have been no reports from Slovenia, a small Central European country, on either the presence of \textit{H. capsulatum} in caves or of bats infected with histoplasmosis (McMurray & Greer, 1979), bat guano samples from five Slovenian bat-inhabited karst caves were screened for \textit{Histoplasma} antigen and the results compared with those of molecular analyses. Additionally, we analysed risk factors for acquiring histoplasmosis, both in a group of 14 speleologists and in five travellers with imported pulmonary histoplasmosis who consulted the Infectious Disease Department at the Clinical Centre in Ljubljana between 2005 and 2016.

**MATERIAL AND METHODS**

**Collection of bat guano and sample preparation**

Five Slovenian bat-inhabited karst caves containing significant deposits of guano in a form of a heap, sampled in 2011-2012, were included in the study. Out of six samples, two samples of bat guano were obtained from Škocjan Caves (Škocjanske jame, E 13.994° N 45.665°) and one sample from each of the following caves: Huda Luknja Cave (Huda luknja, E 15.1743° N 46.4145°); Predjama Cave (Predjama, E 14.1265° N 45.8156°); Spodnja Kleveška Cave (Spodnja kleveška jama, E 15.2334° N 45.9067°); and Turjeva Cave (Turjeva jama, E 13.5046° N 46.2435°). These guano heaps had already been screened for the fungus \textit{Pseudogymnoascus destructans} (formerly known as \textit{Geomyces destructants}), a bat pathogen, and for free-living amoebae during previous studies (Mulec et al., 2013, 2016). The authors did not interact directly with any bats during sample collection. Briefly, in caves, the guano was sampled aseptically from the upper layer of the guano heaps, from zero to five cm in depth, and stored at -20°C until analysed in a laboratory. The age of samples was estimated at the sites of guano collection. “Fresh” guano samples were characterized by intact rod-shaped excrement, indicating the ongoing presence of roosting colonies of bats at the sites. A “recent” age attribution was given to those samples that included some relatively recent rod-shaped excrement over an older guano base. Samples were considered “old” when the guano showed no characteristics of fresh and/or recent rod-shaped excrement, and if no bat colony had been recorded above the guano heap in recent times, usually within a period of a few decades. Basic information on guano is summarized in Table 1.


**Antigen detection in guano**

In the laboratory, 1 g of each guano sample was suspended in 10 ml of sterile RPMI 1640 – Hepes cell culture medium (Sigma, USA) and vortexed vigorously. These biomass suspensions (1:11 [w/v]) were centrifuged for 5 minutes at 4°C and 9200g. The supernatants were collected undiluted and further diluted 10-fold with sterile RPMI 1640 – Hepes, and stored at -20°C pending further analysis. The ALPHA \textit{Histoplasma} Antigen EIA kit (Immy, USA), a commercially available enzyme immunoassay (EIA) for \textit{in vitro} diagnostic use in human serum and urine samples was used to detect \textit{Histoplasma} antigen in the guano supernatants. Results were expressed as EIA Units of \textit{Histoplasma} antigen. Two commercial \textit{Histoplasma}-antigen preparations needed for the determination of anti-\textit{Histoplasma}-antibodies using the complement fixation method, i.e., \textit{Histoplasma}
Myceilia CF Antigen and Histoplasma Yeast CF Antigen (Immy, USA), were included in the test as additional positive controls.

**DNA isolation and molecular analyses of bat guano**

DNA isolated, using the NucleoSpin® Soil kit with SL2 lysis buffer (optimized protocol for guano samples by Macherey-Nagel, Germany), in a previous study (Mulec et al., 2013), has been used in the present analysis. Using the whole-cell DNA, nucleotide sequences of a highly conserved region of the fungal rRNA gene were amplified with two primer sets, i.e., ITS1-ITS4 and ITS4-ITS5 in a control polymerase chain reaction – PCR (http://www.biology.duke.edu/fungi/mycolab/primers.htm).

_H. capsulatum_-specific PCR assays using two sets of specific primers (H-anti3-H-anti4 andMsp2F-Msp2R), the regions of H-antigen precursor and M-antigen genes (Guedes et al., 2003; de Muniz et al., 2010) were performed. PCR was carried out with serially diluted positive-control DNA to determine the analytical sensitivity of the test. Amplification products were analysed on 1% agarose gels, stained with SYBR Safe DNA gel stain (Invitrogen, USA) and electrophoresed at 140 V for 20 minutes.

**Extraction, amplification, and sequencing of DNA from clinical samples**

The serum specimens from four histoplasmosis patients, who did not belong to the group of examined speleologists, were available from the archival serum collection of the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana, and used to extract DNA. Additionally, total DNA was extracted from paired specimens (urine and serum) from three speleologist volunteers who exhibited positive or equivocal Histoplasma antigen assay in urine. DNA templates were obtained by using the automatic commercial isolation kit MagNA Pure Compact Nucleic Acid Isolation Kit I (Roche Diagnostics, Mannheim, Germany) according to the manufacturer’s instructions. For the investigation of fungi-specific DNA in clinical samples, two sets of universal fungal primers were used as described above.

For sequencing, the amplified PCR products of the proper size (= 620 bp) were purified using Exonuclease I (Exo I) and FastAP™ Thermo sensitive Alkaline Phosphatase (Thermo Scientific, Waltham, USA) according to the recommendations of Werle et al. (1994). DNA sequencing reactions were purified using a BigDye XTerminator® Purification Kit (Applied Biosystems®, USA) and sequencing was done in a 3500 Genetic Analyser (Applied Biosystems®, USA). Finally, sequences were analysed using CLC Main Workbench 6.9.1 (CLC bio, Denmark). _H. capsulatum_-specific PCR was performed as previously described.

**Speleologists and analysis of the risk factors for acquiring histoplasmosis**

A questionnaire, which was addressed specifically at speleologists who work and explore caves and come into direct and/or indirect contact with aerosols derived from bat guano, was composed, and distributed to 14 speleologist volunteers. These speleologists professionally work in caves, most of them being active researchers. They were advised to test for histoplasmosis by their employer and physician as they are frequently exposed to work in potentially biohazardous environments. The questionnaire comprised three parts: travel history to known endemic areas and visits to caves in those regions, information about speleological activities, and data on any accidents in the caves that might have led to infection with _H. capsulatum_ (Appendix: Histoplasmosis Questionnaire). Each speleologist was examined by the same physician to assess health status, and screened for the presence of anti-histoplasmin antibodies (in serum) by immunodiffusion (ID) assay (Meridian, USA), and Histoplasma antigen (in serum and urine) by ALPHA Histoplasma Antigen EIA (Immy, USA) according to the manufacturer’s instructions. Urine samples were screened untreated and undiluted; however, serum samples were treated with pronase (Immy, USA) according to the manufacturer’s instructions. The antigen EIA was considered to be negative when the antigen concentration was less than 2 EIA Units, to be equivocal when it was between 2 and 3 EIA Units, and to be positive when the concentration was above 3 EIA Units.

All samples from speleologists were obtained as a part of a preventive health examination recommended by the employer which initially included a complete physical examination and laboratory tests. Since laboratory testing did not include screening for the presence of anti-histoplasmin antibodies, Histoplasma antigen or _H. capsulatum_-specific DNA, further laboratory tests had been requested by the responsible clinician. Informed consent for additional laboratory testing and analysing the data was obtained in writing from all those who completed Histoplasmosis questionnaire. All speleologists and samples were anonymized, no additional sample was taken for the purpose of the study and only speleologist’s age and gender were available to researchers.

**Table 1. List of bat guano collection sites (Mulec et al., 2013, 2016).**

<table>
<thead>
<tr>
<th>Cave</th>
<th>Temperature (°C)</th>
<th>Bats number</th>
<th>Relative guano age / heap volume (l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Huda Luknja Cave†</td>
<td>9</td>
<td>2200</td>
<td>Recent (630)</td>
</tr>
<tr>
<td>Predjama Cave</td>
<td>12</td>
<td>1320</td>
<td>Recent (50)</td>
</tr>
<tr>
<td>Spodnja Klevevška Cave†</td>
<td>18</td>
<td>400</td>
<td>Fresh (30)</td>
</tr>
<tr>
<td>Škocjan Cave</td>
<td>12</td>
<td>5300</td>
<td>Fresh (400), Old (450)</td>
</tr>
<tr>
<td>Turjevica Cave</td>
<td>10</td>
<td>100</td>
<td>Old (800)</td>
</tr>
</tbody>
</table>

†Cave with a thermal spring
Patients and criteria for classification of cases

Five cases of imported pulmonary histoplasmosis, diagnosed at the Infectious Disease Department in the Clinical Centre at Ljubljana between 2005 and 2016 were analysed retrospectively. A review of each patient’s medical record was provided by the treating physician to categorize individuals with histoplasmosis. The cases were classified following the European Confederation of Medical Mycology Working Group definitions (Ashbee et al., 2008). Histoplasmosis was considered probable when the individual had a travel history to a known endemic area, presented positive results to serological tests and where imaging of the lung revealed lesions consistent with histoplasmosis. Cases were classified as possible when the individual had a history of travel to a known endemic area and either showed a positive result to serological tests or if imaging of the lung revealed lesions consistent with histoplasmosis. Since laboratory testing initially included screening for the presence of anti-histoplasmin antibodies, additional retrospective Histoplasma antigen EIA was performed.

With regard to disease presentation, patients presenting with an acute primary infection after recent exposure to H. capsulatum risk factors were classified according to Wheat et al. (2016) as acute pulmonary; patients presenting with milder symptoms than those with an acute infection (symptoms are the same as but milder than those of acute histoplasmosis, with chest imaging showing focal or patchy opacities instead of diffuse bilateral) was classified as subacute pulmonary histoplasmosis. A patient who was asymptomatic, developed subclinical, self-limited, disease.

The serum specimens for additional Histoplasma antigen EIA testing from four histoplasmosis patients were available from the archival serum collection of the Institute of Microbiology and Immunology, Faculty of Medicine, University of Ljubljana. We followed the principles of the Helsinki Declaration, the Oviedo Convention on Human Rights and Biomedicine, and the Slovene Code of Medical Deontology. All samples were anonymized and only patient’s age, gender, geographical destination of travelling, anti-histoplasmin antibodies result, and therapy were available to researchers.

RESULTS

Histoplasma in bat guano

Fungal broad range PCR on total DNA isolated from guano samples confirmed the presence of fungal DNA in all samples. However, H. capsulatum-specific PCR assays using H-antigen and M-antigen primers that displayed the analytical sensitivity of 26.6 pg/µl were negative (Fig. 1). In a direct approach to determine the specific fungus sequence, no sequence related to H. capsulatum was retrieved in any of the bat guano samples.

All six guano samples from Slovenian caves were positive for Histoplasma antigen in both undiluted and 10-fold diluted supernatants, determined by EIA (Fig. 1). The sample of old guano from Turjeva Cave had the highest antigen content. Because of the limited number of samples, the effect of guano age on Histoplasma antigen remains elusive. Because concentrations of Histoplasma antigen were substantially higher in all 10-fold diluted supernatants than in the undiluted ones, unspecific, false positive antigen results in guano samples were suspected. Both commercial antigen preparations (mycelial and yeast phase Histoplasma antigen), tested positive, showing 232.3 EIA Units and 145.4 EIA Units respectively.

A) Cave, relative guano age

<table>
<thead>
<tr>
<th>Primer</th>
<th>Huda Luknja Cave, recent</th>
<th>Predlagna Cave, recent</th>
<th>Spodnja Klevacka Cave, fresh</th>
<th>Skocjan Caves, fresh</th>
<th>Skocjan Caves, old</th>
<th>Turjeva Cave, old</th>
</tr>
</thead>
<tbody>
<tr>
<td>ITS1-ITS4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>ITS4-ITS5</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>H-anti3/H-anti4</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Msp2F-Msp2R</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

B) 1800

Fig. 1. Histoplasma Antigen EIA content in bat guano samples was not supported by the molecular analyses. A) Two universal fungal primer pairs, ITS1-ITS4 and ITS4-ITS5, and two H. capsulatum-specific primer pairs, H-anti3-H-anti4 and Msp2F-Msp2R, were used for the analysis of genomic DNA; B) Histoplasma antigen was measured by the ALPHA Histoplasma Antigen EIA in undiluted (1:11 [w/v]) and in 10-fold diluted (1:110 [w/v]) guano supernatants.
Taken together, Histoplasma Antigen EIA results were not supported by the molecular analyses.

**Epidemiological characteristics and diagnosis of histoplasmosis in speleologists**

A total of 14 speleologists were studied (seven females, seven males). Their mean age was 39.7 years (the youngest was 27 and the oldest 58 years old). Two speleologists reported nonspecific health problems at the time of the visit at our clinic. Otherwise they were all healthy individuals, without any known immunosuppression and with unremarkable medical histories.

Most of the subjects had been active speleologists for a number of years, ranging from 4 to 43 years, with the mean of 17.5 years. Visits to caves once a week were reported in five cases (35.7%), once a month in seven cases (50%) and several times a year for one (7.1%) person. One person didn’t specify the frequency. The speleologists visited caves in different regions of the world. North-American caves were visited by ten (71.4%) of the speleologists, Central-American by six (42.9%), South-American by four (28.6%), African by three (21.4%), Australian by five (35.7%), and Southeast-Asian and Indian caves by ten speleologists (71.4%). Five speleologists (35.7%) reported visiting only touristic caves, 8 (57.1%) also visited other wild caves, and one person (7.1%) didn’t provide an answer. None of the persons complained about health problems when returning from caves.

Protective measures were employed by five persons (35.7%), all measures except for use of a protective face mask were reported by three speleologists (21.4%), whereas six people (42.9%) used no protection.

None of the 14 speleologists reported a bite by a bat. One had an accident in the cave, but with no serious consequence. Known contact with bats or guano were reported by nine (64.3%) of the subjects.

Results of the medical check-up were unremarkable in all 14 speleologists. In spite of having risk factors for acquiring infection with *H. capsulatum*, all speleologists tested negative by immunodiffusion and *Histoplasma* antigen EIA in serum (Table 2). For all but three, *Histoplasma* antigen EIA results in urine were negative. One person showed an equivocal antigen EIA test result in urine (2.0 EIA Units) and two results were low positive (3.9 and 3.2 EIA Units). PCR was performed on serum and urine samples from these three speleologists and results for *H. capsulatum* were negative in all cases (Table 2). The speleologist positive for the *Histoplasma* Ag (3.9 EIA Units) reported in his questionnaire that he visited some of the endemic areas (Northern America, Mexico, India and Indonesia) and he gets occasionally in contact with guano and bats. Broad range PCR revealed the presence of *Cladosporium* sp. in his urine. The second speleologist (3.2 EIA Units) reported visits in North America, India and Malesia, occasional contacts with bat guano, and she had *Malassezia restricta* in her urine and serum.

Table 2. Demographic characteristics of speleologists and results of diagnostic tests for histoplasmosis performed.

<table>
<thead>
<tr>
<th>Speleologist</th>
<th>Sex</th>
<th>Sample</th>
<th>PCR result</th>
<th>ID result</th>
<th><em>Histoplasma</em> Ag result (EIA Units)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Male</td>
<td>Serum</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>2</td>
<td>Male</td>
<td>Serum</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive (3.89)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>3</td>
<td>Female</td>
<td>Serum</td>
<td>Negative</td>
<td>Negative</td>
<td>Positive (3.19)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>4</td>
<td>Female</td>
<td>Serum</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>5</td>
<td>Female</td>
<td>Serum</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>6</td>
<td>Male</td>
<td>Serum</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>Negative</td>
<td>Negative</td>
<td>Equivocal (2.02)</td>
</tr>
<tr>
<td>7</td>
<td>Female</td>
<td>Serum</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>8</td>
<td>Male</td>
<td>Serum</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>9</td>
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<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td>10</td>
<td>Male</td>
<td>Serum</td>
<td>ND</td>
<td>Negative</td>
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<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td>11</td>
<td>Female</td>
<td>Serum</td>
<td>ND</td>
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<td>Negative</td>
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<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
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<td>12</td>
<td>Male</td>
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<td>Urine</td>
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<tr>
<td>13</td>
<td>Female</td>
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<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
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<tr>
<td>14</td>
<td>Female</td>
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<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Urine</td>
<td>ND</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; ID, immunodiffusion test; EIA, enzyme immunoassay; Ag, antigen; ND, not done
Epidemiological characteristics and diagnosis of histoplasmosis in travellers

During the last ten years five cases of histoplasmosis among travellers were diagnosed at the Infectious Disease Department of the Clinical Centre Ljubljana, Slovenia (Table 3). At the time of presentation, based on epidemiological data, clinical symptoms, and X-ray imaging, four patients had a highly-likely suspicion for pulmonary histoplasmosis (patients 1, 2, 4, and 5). The most common symptoms were cough, headache, myalgia, fever, and chest pain. One person was asymptomatic (patient 3) and was traveling with the patient who presented with acute pulmonary histoplasmosis (patient 2). They were all males, with mean age of 37.2 years without any underlying diseases, and with no known immune deficiency. The epidemiological background of patients included travel to Histoplasma-endemic regions. One patient had spent much time doing outdoor activities in National Parks throughout the Mississippi Valley, USA, two patients had collected bat guano for soil fertilizer in non-touristic caves in Jamaica, and two patients had visited rural areas, but not caves with bat guano in Venezuela and Ecuador. None of them was a speleologist. They were not aware of the potential presence of H. capsulatum in the region, and no one adopted any personal protection measures against the pathogen.

On medical examination, the lungs were normal on auscultation in four patients; one patient was displaying signs of pathological compromise of the lungs (patient 1). The rest of examination was not remarkable. Pathological chest radiographs for four patients were consistent with histoplasmosis (patients 1, 2, 4, and 5). Prompted by the pathology seen on the chest radiographs, computed tomography (CT) scans of the chest were performed. Pathology highly indicative of pulmonary histoplasmosis was confirmed in all four patients.

Out of four patients with respiratory symptoms and indicative results of pathological chest imaging, three patients showed positive immunodiffusion results; one patient was seronegative. A patient who was asymptomatic tested positive by immunodiffusion. Out of four seropositive patients, two had the positive antigen EIA test in serum, one was equivocal, and one tested negative. Tests for H. capsulatum-specific PCR were performed on serum samples from four seropositive patients and results were negative in all cases.

In addition, three patients met the criteria for probable histoplasmosis and one patient for possible histoplasmosis. A patient who was asymptomatic and seropositive, had subclinical, self-limited histoplasmosis. In all, three patients presented with acute pulmonary histoplasmosis (patients 1, 2, and 5), of whom two patients were classified as probable and one patient as possible histoplasmosis; therapy with itraconazole was initiated. All three patients received an initial dose of 200 mg of itraconazole three times daily for three days. Thereafter, itraconazole was administered with the dose of 200 mg twice daily for a total of 12 weeks in patient 1, 6 weeks in patient 2 and 8 weeks in patient 5. All three patients responded well to therapy and the pathology seen on the chest radiographs was completely abrogated after several months. The patient with subacute pulmonary histoplasmosis and the asymptomatic one were not treated.

**DISCUSSION**

A small but significant number of autochthonous cases of histoplasmosis reported in European countries, especially in neighbouring Italy (Manfredi

<table>
<thead>
<tr>
<th>Patient</th>
<th>Age</th>
<th>Sex</th>
<th>Travelled to</th>
<th>Sample</th>
<th>PCR result</th>
<th>ID result</th>
<th>Histoplasma Ag result (EIA Units)</th>
<th>Classification of histoplasmosis</th>
<th>Clinical category</th>
<th>Therapy</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>51</td>
<td>Male</td>
<td>USA (Mississippi Valley)</td>
<td>Serum</td>
<td>Negative</td>
<td>Positive (M band)</td>
<td>Negative</td>
<td>Probable</td>
<td>Severe acute pulmonary histoplasmosis</td>
<td>Itraconazole 200 mg TID for 3 days then 200 mg BID for a total of 12 weeks</td>
</tr>
<tr>
<td>2</td>
<td>32</td>
<td>Male</td>
<td>Jamaica (wild caves)</td>
<td>Serum</td>
<td>Negative</td>
<td>Positive (M band)</td>
<td>Positive (3.66)</td>
<td>Probable</td>
<td>Acute pulmonary histoplasmosis</td>
<td>Itraconazole 200 mg TID for 3 days then 200 mg BID for a total of 6 weeks</td>
</tr>
<tr>
<td>3</td>
<td>27</td>
<td>Male</td>
<td>Jamaica (wild caves)</td>
<td>Serum</td>
<td>Negative</td>
<td>Positive (M band)</td>
<td>Equivocal (2.51)</td>
<td>Not classified</td>
<td>Subclinical histoplasmosis</td>
<td>No</td>
</tr>
<tr>
<td>4</td>
<td>39</td>
<td>Male</td>
<td>Venezuela (outdoor activities)</td>
<td>Serum</td>
<td>Negative</td>
<td>Positive (M band)</td>
<td>Positive (3.91)</td>
<td>Probable</td>
<td>Subacute pulmonary histoplasmosis</td>
<td>No</td>
</tr>
<tr>
<td>5</td>
<td>35</td>
<td>Male</td>
<td>Ecuador (outdoor activities)</td>
<td>Serum</td>
<td>ND</td>
<td>Negative</td>
<td>ND</td>
<td>Possible</td>
<td>Acute pulmonary histoplasmosis</td>
<td>Itraconazole 200 mg TID for 3 days then 200 mg BID for a total of 8 weeks</td>
</tr>
</tbody>
</table>

PCR, polymerase chain reaction; ID, immunodiffusion test; EIA, enzyme immunoassay; Ag, antigen; ND, not done; TID, three times daily; BID, twice daily.
et al., 1994; Farina et al., 2000, 2005; Calza et al., 2003; Ashbee et al., 2008), leads us to consider whether *H. capsulatum* might not be confined to only a limited region but might actually be an autochthonous inhabitant of Central Europe, including Slovenia. In the study, the commercial ALPHA *Histoplasma* Antigen EIA kit designed for human in vitro diagnostics of histoplasmosis was applied for the first time to capture *H. capsulatum* antigen from bat guano heaps, not mixed with the surrounding cave material. The 10-fold diluted guano supernatants revealed a substantially higher antigen content than did undiluted supernatants (Fig. 1), implying that conditions in guano might have been the reason for discordant results and probably for non-specific reactions. For example, reactive antigen EIA might be linked to the cross-reactivity of rabbit anti-*Histoplasma* IgG antibodies used in the test with polysaccharide antigens of diverse and abundant guano-associated microorganisms rather than *H. capsulatum* (Chroňaková et al., 2009; Nieves-Rivera et al., 2009). Indeed, our previous study demonstrated that the sampled guanos are rich sources of cultivable fungi and bacteria (Mulec et al., 2013), but species identification was not carried out. Fungi associated with bat guano are diverse (Nováková, 2009). Besides being a source of nutrients and microbiota, guano as a complex environmental sample can also vary in pH, ranging from acidic to alkaline (Mulec et al., 2013) and is known to contain high levels of heavy metals (Walker et al., 2007; Kristšufek et al., 2010), which all together could facilitate the non-specific reactions in the *Histoplasma* antigen EIA.

The PCRs with the primers specific for *H. capsulatum* var. *capsulatum* (Guedes et al., 2003; de Muniz et al., 2010) were performed on total DNA isolated from the guano samples and nucleotide sequences of a highly conserved region of the fungal rRNA gene were analysed. Although the guano samples tested *Histoplasma*-antigen positive, no *H. capsulatum*-specific PCR products were detected and no sequences related to *H. capsulatum* were retrieved from any of the bat guano samples (Fig. 1). The results indicated that the commercial ALPHA *Histoplasma* Antigen EIA kit is probably not applicable for complex environmental samples such as bat excrement.

On the other hand, the negative results of the specific PCRs can point out that concentrations of *H. capsulatum* DNA in the samples were below the detection limit. It might also be that bat species identified in Slovenian caves are not the natural hosts of the fungus, and consequently the fungus is not yet present in guano. Several bat species have been identified in caves in Slovenia; however, the main origin of screened guano was from the bat *Miniopterus schreibersii* (Mulec et al., 2016), which is not yet reported to host *H. capsulatum* (Stanič-Paveličič and Grom 2005). Although the presence of reactive antigen EIA was not supported by the molecular analyses and therefore *H. capsulatum* was probably not present in bat guano, the possibility of transmission of the fungus by a different species of bat in Slovenia cannot be excluded. Further work is needed to screen guano from other caves for the presence of *H. capsulatum* or the association of histoplasmosis in bats.

The increasing number of people travelling to *Histoplasma*-endemic areas is responsible of the growing number of reports of single or, more frequently, clusters of acute histoplasmosis cases. The continued reports of cave-associated outbreaks suggest that current caving practices continue to place cavers at risk from the infection, as a particularly vulnerable population (Senechal et al., 2012; Bahr et al., 2015; Benedict & Mody 2016). By analysing cases of imported pulmonary histoplasmosis in Slovenia retrospectively, our findings, reported here for the first time, suggest that visiting caves in *Histoplasma*-endemic areas and coming into contact with bat guano without the use of personal protective equipment were the major risk factors for acquiring the infection in the travellers group. Although several risk factors for acquiring histoplasmosis were indicated for the speleologists group, e.g., visiting confined underground spaces in endemic areas, contact with the bats or their guano and inadequate employment of protective measures, none of them showed clinical signs and/or symptoms of histoplasmosis at the time of medical examination. We highlight a general lack of awareness of this disease among professional speleologists and other cave explorers, who should use personal protective equipment to prevent infection. For example, two of the professional speleologists from the studied group (speleologists no. 4 and no. 6 in Table 2) did not acquire histoplasmosis during the international cave exploration in Viñales, (Cuba), whereas another speleologist from the international caving group who was not wearing a protection mask, and did not participate in the current study, got histoplasmosis. In the speleologists group, personal protective equipment and/or measures were used only by five persons (35.7%); nine persons (64.3%) did not use protective face masks and six persons (42.9%) did not use any protection. Travelers and not only speleologists who visit endemic areas should be better informed on risk factors for histoplasmosis and its prevention. The contribution of histoplasmosis to travellers’ and cavers’ morbidity is probably underestimated in non-endemic areas (Buitrago et al., 2011; Senechal et al., 2012). Even if it is usually a self-limited illness in immunocompetent individuals, European clinicians should consider it routinely when examining any person with febrile respiratory syndrome who has recently been involved in outdoor activities or visited caves, not only in endemic areas but also in Europe.

Serologic ID assay for the presence of M and H precipitin bands plays an important role in the diagnosis of acute pulmonary histoplasmosis (Kauffman, 2007); however, false-negative results may occur for patients with recent infection. Out of five Slovenian travellers with histoplasmosis, four had solely an M band and one patient was seronegative. It is noteworthy that the seropositive traveller with severe acute pulmonary histoplasmosis tested as *Histoplasma*-antigen negative in serum; however, urine was unfortunately not available for reaching a
areas. Although five imported cases of pulmonary infection, particularly during exploration in endemic personal protective equipment to help prevent among persons exploring caves, who should use the widespread lack of awareness of histoplasmosis by the results of molecular analyses. We highlight samples such as bat excrement was not supported antigen EIA for complex environmental Histoplasma infection has not been established.

In conclusion, we have shown that the reactive Immy Histoplasma antigen EIA for complex environmental samples such as bat excrement was not supported by the results of molecular analyses. We highlight the widespread lack of awareness of histoplasmosis among persons exploring caves, who should use personal protective equipment to help prevent infection, particularly during exploration in endemic areas. Although five imported cases of pulmonary histoplasmosis were diagnosed in Slovenia during the last ten years, we infer from our sequencing results from bat guano and the fact that, to date, there have been no reports of autochthonous histoplasmosis among tourists, speleologists or bat researchers visiting Slovenian caves, that H. capsulatum is probably not present in the caves of Slovenia. On the other hand, histoplasmosis should be considered in a differential diagnosis for a febrile respiratory disease outbreak in returning travellers with a history of geographical exposure.

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Active growth of non-hydrothermal subaqueous and subaerial barite (BaSO₄) speleothems in Lechuguilla Cave (New Mexico, USA)

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Abstract: Barite (BaSO₄) speleothems have been reported from caves around the globe and interpreted to have chiefly formed in phreatic, hypogene, hydrothermal settings. Here we report two contrasting types of barite speleothems (bluish tabular crystals in a shallow pool and actively dripping greenish stalactites), which today form at lower temperatures in the non-hydrothermal and vadose environment of Lechuguilla Cave, New Mexico, USA. Scanning electron microscopy analysis, along with energy- and wavelength-dispersive X-ray spectroscopy (EDS, WDS), as well as X-ray diffraction (XRD), characterize the habit and chemical composition as barite. Fractionation of the minor element calcium is related to growth along different crystal faces whereas variations in strontium concentration are mirrored in blue color zoning of the pool crystals. Two possible modes of non-hydrothermal barite precipitation are discussed: (1) intense evaporation driven by thermal atmospheric convection cells or (2) mixing of barium-rich, sulfate-poor water with water rich in sulfate. Both processes, in isolation or in combination, lead to supersaturation and could explain formation of the investigated barite speleothems. Observations of three types of microbes on the pool barite crystals showing evidence of incrustation raises the question whether there is a potential involvement of microbial activity in the temperate barite precipitation in Lechuguilla Cave.

Keywords: barite, speleothem, precipitation, microbes, incrustation

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INTRODUCTION

The mineral barite (BaSO₄) is best known for its high density (4.5 g/cm³), which has led to its use in a number of economically important applications. Due to its low dissolution rate, barite is generally undersaturated in natural waters, although large quantities can be found in marine settings. There, barite can form in the water column, on the seafloor, or within the sediment, where (1) barium-rich organic matter decays, (2) precipitation is mediated by microbial activity, (3) waters rich in barium and sulfate mix, or (4) hydrothermal fluids rich in dissolved minerals cool down (Hanor, 2000; Griffith & Paytan, 2012).

In continental settings, the formation of barite is usually associated with hydrothermal activity supporting the formation of massive barite as a component of ore deposits (Hanor, 2000) including formation of colorful mineral aggregates (Rustemeyer, 2015). The remobilization and phreatic reprecipitation of barite in a hypogene hydrothermal karst environment is believed to be responsible for the majority of the known occurrences of barite as a cave mineral (for review, see Hill & Forti, 1997). For some of those occurrences, a hydrothermal origin has been demonstrated by analysis of fluid inclusions and/or sulfur stable isotope analyses (for review, see Dublyansky, 1997). For at least one occurrence, the barite replacement boxwork recently reported from the Frasassi caves in Italy (Galdenzi, 2019), a non-hydrothermal origin is conceivable.

Barite speleothems that formed in the vadose zone under subaerial conditions in caves are rare. Occurrences include unique hollow barite helictites of a cave in the Silius barite-fluorite mine, southwest Sardinia (Hill & Forti, 1997), and helictites with a radial arrangement of surficial barite crystallites in Madoc Cave, Ontario, Canada (Walker, 1919). Reworking of hydrothermal mineral deposits by H₂S-
rich condensation waters and redeposition as barite (and celestine) on the surface of calcite speleothems has been reported from the Cup-Coutunn Cave System, Turkmenia (Mal'tsev & Malishesvky, 1990; Mal'tsev & Self, 1992). More recently, barite crystals were detected in rims associated with floor vents that are considered hypogene in Cova des Pas de Vallgornera, Majorca, Spain (Fornós et al., 2010; Onac et al., 2014). Barite stalactites with typical vadose morphology (including a central canal and radial arrangement of crystallites) were reported from Tunisia (Rustemeyer, 2015). Active formation has not been demonstrated for any of these occurrences.

The fact that barite can form in non-hydrothermal settings in the ocean (Griffith & Paytan, 2012), such as cold vent sites (Greinert et al., 2002; Stevens et al., 2015), raises the question whether barite could also form in caves under non-hydrothermal conditions. In this paper we characterize two types of actively growing barite speleothems in a temperate environment (normal-temperature cave setting = 5-25°C sensu Dublyansky, 1997), both discovered in Lechuguilla Cave, New Mexico, USA.

Lechuguilla Cave is among the world’s largest cave systems, with more than 240 km of mapped passages (Lyles & Davis, 2016; Lynch, pers. comm., 2019). The cave formed in carbonate rocks of the Permian Capitan Reef Complex in the Guadalupe Mountain uplift through hypogene sulfuric acid speleogenesis (DuChene, 2000; Hill, 2000a, b). During the Miocene and Pliocene, hydrogen sulfide (H2S), derived in part from microbial activity in hydrocarbon reservoirs in the adjacent Delaware Basin, mixed with oxygenated waters of the karst aquifer to form sulfuric acid (H2SO4) that caused extensive carbonate dissolution and cave origin (Hill, 1987, 2000a, b; Jagnow et al., 2000; Palmer & Palmer, 2000; Barton, 2013; DuChene et al., 2017). Substantial deposits of gypsum (hydrated CaSO4) were left behind as a byproduct and their secondary dissolution and reprecipitation led to the formation of a wide variety of speleothems (Davis, 2000; Palmer & Palmer, 2000; Polyak & Provencio, 2001; Palmer, 2006). Lechuguilla Cave contains a substantial suite of rare cave minerals, many of which are directly or indirectly related to sulfurous acid speleogenesis (Cunningham et al., 1993; DuChene, 1997; Davis, 2000; Polyak & Provencio, 2001). This inventory includes barite derived from the host rock and associated corrosion residues (Spilde, pers. comm., 2019). Near the entrance of Lechuguilla Cave, a deposit of pale blue tabular barite, intergrown with translucent blocky calcite, is thought to be related to local Mississippi Valley Type (MVT) ores that formed in the host rock long before the cave (Hill, 1993); a nearby occurrence, possibly of same origin, was recently noted at the top of the dome ‘Purple Rain’ (Hunter, pers. comm., 2019). Nonetheless, there is evidence of barite as a cave mineral that is currently forming speleothems within the cave. These include unusual greenish, subaerial stalactites that drip water, first identified in 1998 in an area called ‘Frostworks’ (Bosted, 1998; LaForge, 1998; Davis, 1999), and bluish, bladed barite crystals forming at the bottom of a calcite-lined pool in an area called ‘Blanca Navidad’, discovered in 2016 during a resurvey of this part of the cave.

The goal of this paper is to confirm the identity of these barite speleothems and to characterize them using a combination of crystallographic investigation, micro-structure analysis, and elemental mapping. The results confirm the presence of active barite speleothems forming in the cave under low-temperature conditions. Based on our results and circumstantial evidence from the geological and speleological setting, potential modes of barite precipitation are discussed.

**MATERIALS AND METHODS**

**Sampling**

A piece of a broken stalactite from the ‘Frostworks’ location was collected in 1998 near survey station EYW L38 as part of the Lechuguilla Cave Mineral Inventory Project (LCMIP). The sample was stored in a plastic film canister for transport before analysis. One barite crystal was removed in 2016 near station ECK R22B from a pool in ‘Blanca Navidad’, using sterile tweezers and stored in a sterile, 15 ml Falcon tube stabilized by Kimwipes.

**Mineralogical and geochemical analyses**

Samples were air-dried and photographed with a Nikon D700 DSLR camera and AF-S Micro NIKKOR 60 mm 1:2.8 ED lens on a black as well as a white background for characterizing translucence and color zoning. Samples were then mounted on aluminum stubs, coated with gold using a Cressington 108auto sputter coater, and imaged via scanning electron microscopy (SEM) on a Tescan VEGA3 xmu at 20 keV, using secondary electron (SE) and backscatter electron (BSE) detectors. For mineral identification, samples were ground to powder in a mortar. X-ray diffraction (XRD) analyses were carried out on a Philips PW1729 X-ray generator with a 2200 Watt 60 keV Cu X-ray source, monochromized with quartz. Powdered samples were analyzed at 40 keV and 30 mA in a 2 Theta range from 15 to 60°. Results were compared to barite reference spectra archived at www.mindat.org.

For a general element screening the SEMs OXFORD X-Max energy dispersive X-ray spectrometer (EDS) with INCA software (v. 5.05) was used. Spectral analyses were carried out at 10 and 30 keV acceleration voltage by scanning several areas of 100 x 100 μm for 300 seconds, with process time 5 at 10 keV and 3 at 30 keV, using 2000 channels and a beam intensity of 15, resulting in >1,000,000 counts per spectrum.

In preparation for high-resolution elemental mapping and quantification with wavelength dispersive X-ray spectroscopy (WDS), the samples were impregnated in R&G “water clear” epoxy resin in a Struers CitoVav vacuum chamber, trimmed on an Uniprec WOCO50 rock saw, ground on a Buehler Ecomet III grinder/polisher with P400 to P1200 grit SiC paper, polished on a ‘G’ cloth with 1 and 0.3 μm MicroPolish powder (all Buehler), cleaned in an...
Elma Transsonic Ti-H-5 ultrasonic bath, rinsed with de-ionized water, air dried, and carbon coated with a Vacuum Coating Unit E306A of Edwards. For the WDS analyses a JEOL Superprobe JXA-8200 electron microprobe was utilized. Elemental maps (C, O, S, K, Ca, Fe, Co, Ni, Zn, Sr, and Ba) were produced on a 5 and 10 µm grid for overviews of the ‘Blanca Navidad’ and ‘Frostworks’ samples, respectively, with a 10 µm probe diameter at 100 ms dwell time, 15 keV acceleration voltage and a beam current of 100 nA. Detail maps of up to 200 x 200 µm in area were acquired on a 1 µm grid at the margin of the crystals, with a 1 µm probe diameter at 100 ms dwell time, 15 keV acceleration voltage and a beam current of 100 nA. For those five elements (O, S, Ca, Sr, and Ba) that returned a signal other than background noise (X-ray bremsstrahlung), quantitative transects were logged in 10 and 5 µm spacing across overview maps, and 1 µm spacing across detail maps, applying 5 or 1 µm probe diameters, respectively, at 15 keV acceleration voltage, 50 nA beam current, and 20 s peak / 10 s background position time for each element. The detection limits were ~200 ppm (O), ~50 ppm (S), ~40 ppm (Ca), ~160 ppm (Sr), and ~240 ppm (Ba). Reference standards were barite (O, S, Ba), dolomite (Ca), and strontianite (Sr). Measurements with more than 3 % deviation from 100 (total) weight % were excluded from further analysis, as they reflect epoxy resin, embedded impurities, or cracks (22 out of 799 measurements in the ‘Frostworks’ sample and 15 out of 1764 measurements in the ‘Blanca Navidad’ sample).

**RESULTS**

**Field description and mineralogical analysis of barite stalactites**

All of the six sites in Lechuguilla Cave with confirmed or suspected barite stalactites are located within 500 m of each other in the western branch of the cave (Fig. 1).

These sites include a tall room called ‘Frostworks’, with aragonite frostwork and calcite crystals on the bottom and corrosion residues that coat the walls and ceiling, reflecting evaporation and condensation, respectively, in a local convection loop. Near the ceiling (survey stations EYW37 to 38), there are stalactites up to 20 cm in length, partly wavy to angled, composed of pale translucent greenish barite (Fig. 2A-B). The stalactites have a platy structure with parallel to sub-parallel sheets running lengthwise and showing flat cleavages or crystal faces. These speleothems are partly covered with a white calcite crust and rhombohedral calcite crystals. All stalactites are located at low points of hanging breakdown blocks and have moist surfaces. Shortly after this discovery, a sample was taken and a preliminary examination of a powdered subsample via XRD analysis confirmed that the sample is barite (DuChene, 1998). The remainder of that sample is used for analysis here.

Additional greenish stalactites are found in ‘Cephalopodunk’ at station IJ17 (Allison, 2002). The site is located below a breakdown maze with orange/rusty corrosion residue, in an alcove decorated with aragonite frostwork. Two light green stalactites are active and dripping and similarly colored tabular crystals are found at the splash point below (Fig. 2C) (Lyles, pers. comm., 2018). More stalactites are found in ‘Chandelier Graveyard’. One of these, near station EYE26A, is slightly more bluish in color, 20 cm in length, inclined about 10° from vertical, and also dripping water (Fig. 2D-E). A greenish-bluish splash point is forming on the floor below (Fig. 2F). A nearby stalactite at station EGAB8 is pale greenish in color, approximately 15 cm long, oriented about 5° from vertical, and partly covered by white aragonite or calcite crystals (Fig. 2G).
Our sample of a ‘Frostworks’ stalactite (Figs. 2H-K, 3A) is composed of an aggregate showing parallel crystallites resulting from parting, tabular to the pinacoid base $c\{001\}$ in the orthorhombic system (see Fig. 4 for a diagram of the crystallographic properties). This is the face with most perfect cleavage (Fig. 2I, K) and was probably oriented parallel to the axis of stalactite growth. The outer surface of the stalactite primarily shows faces of the prism $m\{210\}$ and pinacoid $a\{100\}$ (Figs. 2H, 3A), and subordinate combinations with prism faces $d\{101\}$ and $o\{011\}$ (Figs. 3A-C). In SEM images, crystal faces appear smooth and homogeneous in SE and BSE images (Figs. 3B-D, F-I), but there are signs of intergrowth with siliciclastics (Fig. 3B-E), such as quartz and clay minerals (‘q’ and ‘c’ in Fig. 3E). Some round patches of clay are found on the surface (Fig. 3F-H), and impurities are found also deeper inside (Fig. 3I). Voids are present where

![Fig. 2. Greenish to bluish stalactites in Lechuguilla Cave. A) Suspected barite stalactite, about 15 cm long, in ‘Frostworks’; B) Another stalactite, found broken on the floor of ‘Frostworks’; C) Actively dripping suspected barite stalactite and greenish splash zone below, found in ‘Cephalopodunk’; D-E) Suspected barite stalactite in the ‘Chandelier Graveyard’ and more bluish in color; F) Close-up of bluish splash zone forming on the floor below the stalactite shown in D-E; G) Another suspected barite stalactite in the ‘Chandelier Graveyard’ with partial calcite/aragonite overgrowth; H-K) The analyzed barite sample taken from a broken ‘Frostworks’ stalactite.](image-url)
impurities have disintegrated (Fig. 3F). Although some of the embedded mineral aggregates appear elongate and cylindrical (e.g., Fig. 3D), no obvious biosignatures were identified to suggest the former or contemporary presence of microbes.

Mineral identification via XRD analysis in the 15° to 60° (2θ) range matches the peak signature of barite (Fig. 5A). No other mineral was detected in the sample. A series of EDS spectra of the cross section and surface of the sample show the elemental composition to be chiefly oxygen (O), sulfur (S), and barium (Ba) (Fig. 5B). In addition to these main elements, the La1 peak of the minor element strontium (Sr) was picked up by the peak detection algorithm of the INCA software in some of the spectra. Other trace elements that can substitute for Ba in barite (Ca, K, Ra, or Pb, and more rarely Fe, Cu, Zn, Ag, Ni, Hg, or V; Hanor, 2000; Griffith & Paytan, 2012), were not detected. Spot spectra of mineral aggregates intergrown with the barite correspond to SiO2 (quartz; see ‘q’ in Fig. 3E), and unidentified clay minerals (see ‘c’ in Fig. 3E and the round aggregate in Fig. 3G-H).

High-resolution element mapping via WDS shows a homogeneous distribution of the major elements Ba, S, and O, in a stoichiometric relationship corresponding to that of barium sulfate (BaSO4), thus confirming that the crystal is barite (Fig. 6A; Table 1). This applies also to the outer margin of the crystal, which was mapped and profiled with 1 µm resolution (Fig. 6D), thereby excluding the presence of a detectable layer of wetherite (BaCO3). The only trace elements that were detected are calcium (Ca) and strontium (Sr) (Fig. 6B, E; Table 1). Their occurrences are confined to growth zones near the center of the stalactite where concentrations reach up to 736 ppm Ca and 473 ppm Sr. These zones are oriented parallel to the presumed stalactite growth increments formed primarily by radial growth along the faces of the prism m {210} and pinacoid a {100} (Fig. 6B). The outer portion of the stalactite has very low concentrations of Ca and no

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Fig. 3. Micro-morphology of a ‘Frostworks’ barite stalactite revealed via SEM. A) Overview of the sample from two perspectives; arrows point out the location of B to I; B-C) Small crystallite resulting from parting (SE and BSE image); D) Elongate mineral aggregate embedded in the barite; E) Partially embedded grains of quartz (q) and a clay mineral (c); F) Irregular voids in the crystal; G-H) A round spot of clay minerals on the surface (SE and BSE image); I) Section of the sample showing several embedded impurities.

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Fig. 5. Mineral identification via XRD analysis in the 15° to 60° (2θ) range matches the peak signature of barite (Fig. 5A). No other mineral was detected in the sample.
Fig. 4. Diagram with the mineralogical properties of the barite speleothems sampled in Lechuguilla Cave. The crystallographic axes and principal faces are shown for an idiomorphic tabular crystal prismatic to the pinacoid base c {001}. Parting occurs parallel to c {001} and color zoning (Blanca Navidad pool crystals only) parallel to the prism faces m {210}. High growth rates along the m {210} and a {100} faces (the tabular crystal growing in size) are mirrored in low Ca concentrations, whereas very slow growth along m {210} (the tabular crystal growing in thickness) leads to relatively high Ca concentrations.

detectable Sr (Fig. 6E–F). Calcium shows the highest concentrations (up to 1,888 ppm) in areas of lateral growth, i.e., where radial growth along the prism base c {001} and the combined prism faces d {101} or o {011} has filled open space between radiating tabular crystallites (Fig. 6B & Fig. 4). In consequence, Ca/Sr ratios cluster according to the respective crystal faces (Fig. 6C).

Field description and mineralogical analysis of barite pool crystals

The ‘Blanca Navidad’ area (Fig. 1) consists of an ascending main corridor that leads to a series of steeply inclined passages with walls heavily coated with dark corrosion residues. The main passage is occupied by a large ‘glacier’ of massive gypsum and terminates about 170 m below the elevation of the entrance. The area contains a rich inventory of speleothems and cave minerals, including gypsum (rims, chandeliers, negative splash rings, drip holes), calcite (flowstone, pearls, stalactites, soda straws, draperies, popcorn, subaqueous helictites), aragonite (frostwork, trees, trays, anthodites), and several occurrences of massive elemental sulfur (Davis, 2000 and own observations). The bluish barite pool crystals are found at station ECKR22B in a N-S trending side passage (Fig. 7), a few meters from the ‘Gripping Hand’, a stalactite/draper group with a pool containing subaqueous helictites, both believed to have formed via the common-ion-effect (Davis, 2000). The pool is less than a square meter in diameter and a few centimeters deep (Fig. 7A). It is lined with whitish to pale gray calcite pool spar, in parts forming indistinct shelfstone. Above the water line, pool spar grades into blocky to triangular popcorn and microcrystalline crusts coating the walls. Water feeding the pool seeps in along the calcite-coated wall and drips from active stalactites (e.g., Fig. 7A on the right). The outlet of the pool leads into a canyon with a stream of wet flowstone on its bottom (Fig. 7A to the left). There is no visible water flow.
In the pool, there are several hundred bluish tabular crystals, up to 2 cm in size, commonly showing some parting. The most conspicuous cluster (Fig. 7B-C) is partly isolated from the rest of the pool by sills of calcite pool spar. The color of the transparent crystals ranges from pale brown to pale gray to a bluish tinge (Fig. 7H). More of the gray to bluish crystals were found growing directly on the wet calcite flowstone that runs into the pools (Fig. 7I). In several places above the flowstone, there are massive deposits of gypsum featuring dissolution drip holes. Water that makes contact with this gypsum enters the pools in a discrete flow, where subaqueous helictites have formed. A few meters above the suspected barite speleothems, a deposit of bright yellow elemental sulfur (S8) was discovered (Fig. 7J), some of which are covered in a gray (microbial?) paste (e.g., Fig. 7J front right). Parts of the sulfur are exposed to dripping water and dislocated into the gypsum drip holes or surrounding splash rings. These chunks of sulfur are darker in color and rounded.

Most recently, a new passage located directly above the barite pool was explored and found to contain more (suspected) barite speleothems of interest. This passage – now called ‘Barite Boulevard’ (survey stations EXHA1 to 7) – is occupied by an active (wet) cascade of calcite flowstone with several small pools, draining into the ‘Lake of Liquid Sky’. One of these pools contains pool fingers (microbial filaments incrusted in pool spar) and pale gray to blue suspected barite crystals of the same type as in the sampled pool (Fig. 7F-G), while another pool contains similar crystals with a yellow tinge (Fig. 7H).
Fig. 7. Barite pool crystals in Lechuguilla Cave. A) Calcite-lined shallow pool containing barite crystals, located in a side passage of ‘Blanca Navidad’; the white arrow points to the barite crystal that was collected; the red arrow indicates the cluster of crystals shown in B-C; B) Cluster of barite crystals, some bluish in color, some pale gray; C) Close-up of B, showing the barite crystals with the most intense bluish color zoning; D-E) The sampled barite crystal in incident light on a black and a white background (larger fragment to the left of dashed line later investigated with SEM, EDS, and WDS; smaller fragment used for XRD); F-G) Overview and close-up of a calcite-lined pool in ‘Barite Boulevard’, with pool fingers and with bluish crystals in the corners; H) Yellowish crystals formed where discrete inflow from the flowstone cascade enters another pool; I) Bluish crystals found growing directly on the active calcite flowstone; J) Deposit of elemental sulfur on a block of gypsum located only few meters above the flowstone cascade and pools shown in F-I.
The XRD spectrum of the bluish crystal, in the scan between 15° to 60° (2θ), is compatible with that of barite (Fig. 9A). A small amount of calcite was detected, stemming from the intergrowth with pool spar at the base. Two peaks are artifacts from the aluminum carrier, due to the small amount of sample.

EDS spectra of the cross section and outer surface of the sample determine the elemental composition as chiefly O, S, and Ba (Fig. 9B) in a stoichiometric relation close to that of BaSO₄. In addition, only the Lα₁ peak of strontium was detected.

High-resolution WDS element mapping shows a homogeneous distribution of the major elements Ba, S, and O, in a stoichiometric relationship corresponding to that of barium sulfate, confirming the identification as barite (Fig. 10A, G; Table 2). As in the stalactite sample, mapping and profiles on a 1 µm grid showed that the elemental ratios remain consistent towards the margin of the crystal (Fig. 10D, J), thus excluding the presence of a detectable layer of witherite. Calcium (Ca) and strontium (Sr) were the only detected trace elements, showing a distinct zoning aligned with the direction of growth along the prism m⁻²¹₀ faces, reaching concentrations of up to 4,820 ppm and 26,200 ppm, respectively (Fig. 10B-C, E-F, H-I, K-L; Table 2). Compared to the 'Frostworks’ stalactite, these concentrations are one to two magnitudes higher for Ca and Sr, respectively. The section mapped perpendicular to the c 001 face identifies dominant growth along the prism m⁻²¹₀ faces and subordinate growth on (but parting along) the c 001 faces (Fig. 10G-L). Calcium concentrations are higher in parts that grew along the pinacoid face c 001 (Fig. 4), whereas Sr was found relatively depleted in these zones, thus showing an inverse pattern (compare Fig. 10B-C and K-L). The respective Sr/Ca ratios cluster accordingly (Fig. 10C, L). Average Sr/Ca ratios are 16.6 ± 12.4 (n = 1,097) for crystal growth on the m⁻²¹₀ faces, and 1.6 ± 1.6 (n = 652) for growth along the c 001 faces. There is no difference in concentrations between the neighboring m⁻²¹₀ faces (Fig. 10B) or between the opposite c 001 faces (Fig. 10H). Concentrations of Sr mirror the blue color zoning in that the outermost (youngest) zone shows the highest Sr concentrations and most intense coloration (compare Figs. 10C and 7E).

Three principal types of microbial morphology were identified on, and partly embedded within, the sampled barite crystal:

Type 1 microbes form filaments 300-400 nanometers in diameter and were found collapsed on the surface of the crystal (Fig. 11B-C). The tubular filaments exceed
100 µm in length, have a constant width and are partly flattened by desiccation. Although they are commonly touching or overlapping (Fig. 11B-C), it is unclear whether they represent branching cells. Coccoidal cells or spores ~1 µm in diameter, were seen associated with the filaments (marked as ‘c’ in Fig. 11B). Some filaments enter or exit deep trenches in the crystal, or they disappear into (or emerge from) angular holes (Fig. 11B-E; marked ‘b’ in B) formed by crystal overgrowth.

The Type 2 microbes do not appear to have remained on the surface of the barite crystals after collection/processing. As such, their presence and morphology are only evident where the barite has grown around the original organic filaments, leaving incrustations that mimic their structure. The incrustations suggest filaments that were 200-400 nm in diameter and meander on the surface of the crystal (Fig. 11F). They commonly touch or overlap but unequivocal branching points were not observed. The filaments appear to have originated from a circular central area about 3-10 µm in diameter (Fig. 11F-I; marked by arrows in F) that has an irregular margin with minute protrusions (Fig. 11G). The presence of individual furrows (arrows in Fig. 11H) or the entire structure (Fig. 11I) being partially overgrown by younger barite crystallites, along with barite precipitating between filaments to form micro-terraces that are different in elevation on either side of the filaments (Fig. 11F-I), supports the incrustation of these microbes.

Evidence of Type 3 microbes are empty incrustation structures observed in broken sections of the crystal (Fig. 11J-K). These casts are rod-shaped, circular in cross section, and appear narrower in the center. Individual cavities are three to five microns in length and about one micron in diameter, which is a common size for dividing bacteria.

DISCUSSION AND CONCLUSIONS

Mineralogy and origin of color

The EDS/WDS and XRD analyses identify the elemental composition and mineralogy of the sampled greenish stalactite and bluish pool crystal as barite. This is in agreement with the observed tabular shape and orthorhombic set of faces with a dominance of the pinacoid base {001} and prism faces {210}. In both settings, the barite primarily grows along the {210} faces, whereas precipitation on the {001} face is limited (Fig. 4). As the crystals grow outward from their base at the bottom of the pool, or from the central canal of the stalactite, parting along the {001} faces increases the width of the tabular crystal aggregates.

The only minor elements detected, mapped and quantified via WDS analyses in the present samples are calcium (Ca) and strontium (Sr), which both substitute for barium in barite (Griffith & Paytan, 2012). The color of minerals and the speleothems they form is commonly a result of such minor or trace elements incorporated in the crystal lattice (White, 1997). Strontium is the most common impurity (apart from radium) in natural barite (e.g., Monnin & Cividini, 2006; Griffith & Paytan, 2012) and Sr concentrations have been related to zonal blue coloration by Gaškov.
Fig. 10. A selection of WDS maps (same color scaling as Fig. 6) and profiles of the ‘Blanca Navidad’ pool crystal, showing the crystallographic properties, the position of the profiles, and the distribution of the major elements Ba, O, and S (A, D, G, J) as well as the precipitation patterns reflected in the trace elements Ca and Sr (B-C, E-F, H-I, K-L).
et al. (2017), who reported higher concentrations in darker blue growth zones compared to light colored zones in speleothems from Estonia. This pattern is in good accordance with the ‘Blanca Navidad’ pool crystal where the outermost (youngest) growth zone has the highest Sr concentrations and most conspicuous blue coloration (Fig. 4). The calcium distribution in the same sample, in contrast, is independent of the color zoning. Because no other trace elements were detected in our screening, we consider Sr the most plausible cause of the bluish coloration of the ‘Blanca Navidad’ pool crystals.

The origin of the less distinct and more homogeneous greenish coloration in the ‘Frostworks’ stalactite remains unknown. Sr was found occurring in much lower concentrations and only near the center of the stalactite, and the heterogeneous distribution of Ca likewise does not match the observed coloration. Other trace elements that may substitute for Ba and could cause the coloration were not detected.

![SEM images of microbes and barite incrustations found on and in the sampled ‘Blanca Navidad’ barite pool crystal. A) Overview from three perspectives; arrows point out the location of images in B to K; B-C) Filaments of Type 1, collapsed on the surface, associated with coccoidal aggregates (marked ‘c’) and emerging from angular holes (marked ‘b’), visualized using SE and BSE; D-E) Examples of partially collapsed tubular filaments emerging from holes on the crystal’s surface; F) BSE image of barite incrustations formed around meandering Type 2 filaments running along the surface of the crystal and radiating from a central area (arrows); G) Close up of F, showing circular central area; H) Evidence of partial to complete (arrows) incrustation; I) Angular view of terraced incrustations and large barite crystallites overgrowing the radiating incrustations; J-K) Overview and close-up of rod-shaped Type 3 microbes as recorded by empty incrustations in a broken face.]

Fig. 11. SEM images of microbes and barite incrustations found on and in the sampled ‘Blanca Navidad’ barite pool crystal. A) Overview from three perspectives; arrows point out the location of images in B to K; B-C) Filaments of Type 1, collapsed on the surface, associated with coccoidal aggregates (marked ‘c’) and emerging from angular holes (marked ‘b’), visualized using SE and BSE; D-E) Examples of partially collapsed tubular filaments emerging from holes on the crystal’s surface; F) BSE image of barite incrustations formed around meandering Type 2 filaments running along the surface of the crystal and radiating from a central area (arrows); G) Close up of F, showing circular central area; H) Evidence of partial to complete (arrows) incrustation; I) Angular view of terraced incrustations and large barite crystallites overgrowing the radiating incrustations; J-K) Overview and close-up of rod-shaped Type 3 microbes as recorded by empty incrustations in a broken face.
In both barite speleothem types we see a fractionation in the substitution of Ca and Sr that is related to growth along different crystallographic faces (Fig. 4). Ca was preferentially incorporated during growth along the c {001} prism faces, whereas Sr concentrations are higher in zones grown along the m {210} pinacoid faces. The reason for this fractionation could be grounded in the differing growth rates. Growth along the m {210} faces is much faster compared to the c {001} faces – and results in the tabular habit of the crystals. Or, physical properties of the crystal lattice might facilitate substitution by elements of different ionic radius. In any case, the present findings indicate that the observed fractionation needs to be taken into account when analyzing and interpreting variations in trace element composition of barite speleothems.

**Formation of barite stalactites**

The classical interpretation of barite speleothems as a result of phreatic hydrothermal activity can be excluded for the confirmed and suspected barite stalactites in Lechuguilla Cave. Instead, these stalactites and the greenish/bluish splash zone found beneath some of them, must have formed under subaerial conditions.

The stalactites have moist surfaces and actively drip water, showing that ongoing growth is possible. While we cannot provide proof for such active growth, the fact that the supporting bedrock is still intact (compromised in case of condensation corrosion) and the splash zones have not been overgrown by carbonate precipitates (as in the case of carbonate-saturated alkaline waters) suggest that the speleothems are a relatively recent phenomenon and are likely to still be growing.

These observations support non-hydrothermal growth in the present cave environment. Atmospheric temperatures in Lechuguilla Cave are relatively constant all year around, ranging from 17.3°C near the entrance to 20.4°C at the deep point in the cave (Northup et al., 1994), thus constituting a normal-temperature cave setting (5-25°C sensu Dublyansky, 1997).

Two different modes of non-hydrothermal mineral precipitation need consideration in explaining the formation of the barite stalactites: 1) evaporation, or 2) mixing of water rich in barium with water rich in sulfate. Both processes, in isolation or in combination, could ultimately lead to supersaturation and precipitation.

1) Prominent thermal atmospheric Rayleigh-Benard convection cells in Lechuguilla Cave have long been identified as drivers for condensation and evaporation, controlling the formation of corrosion residues / ferromanganese deposits and leading to directional growth of speleothems, respectively (e.g., Jones, 1990; Queen, 1994; Davis, 1999, 2000; Spilde et al., 2005). In some pools, intense concentration by evaporation results in brine with SO$_4^{2-}$ levels as high as 25,175 mg/l (Turin & Plummer, 2000; Levy, 2008). In ‘Frostworks’, corrosion residues at the ceiling and aragonite frostwork on calcite popcorn at the bottom, together with the observation of significant airflow in the area, suggest that condensation and evaporation take place (LaForge, 1998). This relationship led LaForge (1998) to hypothesize that condensation of upward-moving humid air has resulted in condensation-corrosion and uptake of minerals from the bedrock or corrosion residues, which were then carried by capillary flow to regions where evaporation caused the barite stalactites to crystallize. In support of this idea, all suspected or confirmed barite stalactites are located at low points in the ceiling. In ‘Cephalopodunk’ and the ‘Chandelier Graveyard’ the splash zones on the floor below are additional sites of greenish-bluish mineral formation, where evaporation of the dripping water is supported by less humid, descending air moving along the floor (Queen, 1994). Considering the poor solubility of barite in water (2.2 mg/l at 18°C; Seidell, 1940), this scenario requires immense amounts of water to evaporate (without other evaporates to precipitate) to form barite speleothems the size of the documented stalactites.

2) The alternative model is mixing of waters from two different sources, one rich in Ba$^{2+}$ and devoid of SO$_4^{2-}$, the other rich in SO$_4^{2-}$. Mixing of such waters would result in supersaturation with respect to barite and instant precipitation until saturation is reached (Hanon, 2000; Griffith & Paytan, 2012). In Lechuguilla Cave, this scenario is conceivable where condensation water meets percolating meteoric water, or at mixing points of meteoric waters with different pathways. An obvious source of sulfate is contact with the abundant secondary gypsum deposits, whereas barium ions might derive from the host rock, corrosion residues, ore deposits, or other sources to be identified.

The role of these two models in the formation of the barite stalactites requires more investigation with a focus on different local water sources and their chemistry.

We do not know when the stalactites started to form, but according to Hill (1987, 2000a) subaerial speleothem formation in Guadalupe caves is thought to have commenced in two main episodes in the late Miocene 25-12 Ma ago (Barker & Pawlewicz, 1987) to present day ~20°C/km.

**Formation of barite pool crystals**

We have characterized different types of unidentified microbes on the sample from the ‘Blanca Navidad’ pool. These organic filaments were partly embedded via crystal growth, resulting in incrustations, which suggests that the barite crystals are actively growing today. The micro-topography of the incrustations, including partial overgrowth and stepped terraces with walls at only one side of the microbial filaments, cannot be explained by microbial biocorrosion. Moreover, microbiocorrosion of the highly insoluble barite would require the microbes to produce organic chelators similar to EDTA or DTPA (Wang et al., 2000) or concentrated sulfuric acid (O’Neil, 2013) in large enough quantity to compensate for the buffering capacity of the pool water. Microbially mediated barite
dissolution has been reported in sulfate-limiting conditions (e.g., Phillips et al., 2001) and anoxic brines only (Ouyang et al., 2017), forming simple etch pits different from the complex incrustations observed in our material.

Therefore it appears reasonable that the barite crystals in the ‘Blanca Navidad’ pool are still growing today. The pools with similar crystals in the nearby ‘Barite Boulevard’ are part of an active flowstone cascade with a discrete flow of water and bluish crystals growing on top, thus providing further indication that these speleothems are actively growing today in the present non-hydrothermal, normal-temperature cave environment (5-25°C sensu Dublyansky, 1997). Maximum pool water temperatures in Lechuguilla Cave were determined as 20.0 (Northup et al., 1994) and 20.3°C (Turin & Plummer, 2000, Supplementary Data). Hence, the same two modes of non-hydrothermal mineral precipitation as outlined for the barite stalactites could apply.

1) Recharge and discharge of water into the sampled pool is via discrete seepage from flowstone, complemented by occasional drips from overlying stalactites, so that the retention time in the pool is considerable. This part of ‘Blanca Navidad’ is rich in aragonite frostwork and other speleothems that are indicative of surface diffusion and evaporation. This includes calcite pool spar grading into blocky to triangular calcite popcorn and into microcrystalline crusts coating the walls above the barite pool. On the basis of these signs of evaporation, it is conceivable that evaporation supports the precipitation of the barite crystals. This agrees with the highest density of crystals found in a shallow sub-basin, opposite the point of incoming seepage, filled with overflow water of crystals found in a shallow sub-basin, opposite the point of incoming seepage, filled with overflow water.

2) Mixing of waters of different Ba²⁺ and SO₄²⁻ ion load is conceivable for the sampled pool, given an influx of water via seepage and by droplets from stalactites. Passages higher up in the same fault contain deposits of massive gypsum as a possible source for SO₄²⁻ ions. Nearby subaqueous helictites and the ‘Gripping Hand’ formation, both believed to have formed via common-ion-effect (Davis et al., 1991), support this. mixing of waters and the common-ion-effect are even more evident in ‘Barite Boulevard’, where gypsum deposits with dissolution drip holes are located above the active flowstone cascade and subaqueous helictites are forming in the water bodies directly below. Potential sources of Ba²⁺ ions in percolating meteoric waters are again diverse. Targeted water samples are needed to further evaluate the ion load of the involved sources of water.

The discovery of microbes associated with the barite pool crystals (we cannot exclude their presence in the case of the stalactites) raises the question whether there could be microbial involvement in the precipitation of these barite speleothems, or whether the documented association is coincidence. There is a growing body of evidence that microbial activity is an integral component of barite formation, particularly in the marine realm (Stevens et al., 2015 and references therein), but also in terrestrial settings (Senko et al., 2004). Some bacteria are able to form barite within their cells (e.g., Gooday & Nott, 1982), whereas other bacteria indirectly mediate barite precipitation by serving as nucleation sites for barite precipitation or by means of sulfur/sulfide-oxidation (e.g., Gonzalez-Muñoz et al., 2003, 2012; Stevens et al., 2015; Martinez-Ruiz et al., 2018). In the latter context, the recent discovery of massive gypsum and a substantial deposit of elemental sulfur only few meters above the barite speleothems in ‘Barite Boulevard’ is a puzzling finding that may hold a key for the understanding of the barite precipitation below in terms of microbial sulfur-oxidizing activity. More research is needed to verify whether there are systematic barite-microbe associations, including molecular genetics to identify these microbes and laboratory cultivation experiments to explore their potential involvement in the formation of barite speleothems in Lechuguilla Cave.

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Barite speleothems in Lechuguilla Cave


Uncertainties associated with the use of erosional cave scallop lengths to calculate stream discharges

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Abstract: Scallops are extremely valuable indicators of past water flows in caves because they often record events that cannot be safely witnessed nor measured. Qualitatively, the inverse relationship between their lengths and formative water velocities is useful for determining how flow changes along a cave passage, but they are most valuable because they can be used to directly estimate actual water velocities and discharges. We explore the effects of sample size, measurement choices, and other methods commonly applied to the use of cave scallops in estimating cave stream velocities and discharges. We measured 100 scallops on a cave wall and find them to be log-normally distributed. We used Monte Carlo simulations to sub-sample the 100 scallops for sample sizes of 10 to 30. As expected, smaller sample sizes yield widely varying means with precision increasing slowly with sample size. A sample size of 30 results in greater than 50% of simulated means falling within one standard deviation of the mean for all 100 scallops. This is also true of sample sizes as small as 20, so we recommend a minimum of 20 to 30 scallop measurements in the field. The formulas we use to estimate water velocities and discharges explicitly use the Sauter mean of scallop lengths, but some authors use the arithmetic mean. We simulated the use of both the Sauter and arithmetic means and find that the latter yields substantially larger velocities and discharges. We recommend use of the Sauter mean because that is consistent with the original formulations and the arithmetic mean may cause significant overestimation of velocity and discharge.

Keywords: cave, scallops, karst hydrology, stream, water

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INTRODUCTION

The largest flows or floods often determine or shape stream channel properties (Wolman & Miller, 1960) and large floods are typically the most effective at dissolving and abrading the walls of caves (Gale, 1984; Palmer, 2007). But the largest flows are often short-lived and difficult to measure. Fortunately, caves often sport scallops, which are asymmetrical depressions that record flow velocities. These unique features have been extensively used to understand cave hydrology using scallop lengths measured in the field (Curl, 1974; Palmer, 1976; Lauritzen et al., 1985; Springer & Wohl, 2002; Palmer, 2007; Woodward & Sasowsky, 2009; Despain et al., 2016). However, field-based data always come with uncertainties, but the magnitude and sources of those uncertainties are not always clear or known.

Sampling methods give rise to uncertainties that can be quantified using various statistical measures, including the standard error. The latter, also called the margin of error, can be minimized by taking large samples, but how large is large enough? And there is always the question of sampling biases, which include questions about how individual objects were chosen for measurement and whether the probability of selecting an individual object was the same as the probability of choosing adjacent objects (Davis, 2002). Also, measures of many geological phenomena are not normally distributed, which can limit the uses or statistical analyses of such data, so the shape of the parent population is of interest (Limpert et al., 2001). We explore some of these sources of uncertainty when using cave scallops to estimate floodwater velocities and discharges (Curl, 1974).

Scallops are intricately tiled depressions on solid substrates created by fluid-mediated erosion (Figs. 1 and 2). Scallops are erosional phenomena created by corrosion, ablation, and abrasion. Individual scallops form where a sublaminar jet detaches from a surface, destabilizes into turbulence at some distance downstream, and reattaches to the surface...
The continuity equation, whereby the discharge discharges of Lauritzen (1982) were consistent with Jeannin (2001) using spring discharges using scallop-derived discharges (Lauritzen et al., 1985). Jeannin’s (2001) results were consistent with those percentiles. Lauritzen (1982) concluded that his scallop-based discharges have a precision of ±5.8% and, notably, he reported approximate error ranges based on the standard deviation of scallop lengths (further discussed below).

The reliability or validity of other reported scallop-derived velocities and discharges are difficult to assess because of differences in the assumptions and methods used. These differences extend to such basic decisions as which scallops to measure and what statistical mean to use in the equations of Blumberg & Curl (1974) and Curl (1974). The former authors used the Sauter mean \( \bar{L}_{32} \), which is calculated as:

\[
\bar{L}_{32} = \frac{\sum \bar{L}^2_i}{\sum \bar{L}^2_i} \pm \left( \frac{L_{32} \cdot e^{s_{32}^2} - L_{32}}{L_{32} - \frac{s_{32}^2}{e^{s_{32}^2}}} \right) \tag{1}
\]

where \( L_i \) is the length of the \( i \)th scallop and \( s_{32} \) is the corresponding standard deviation (Lauritzen, 1982). The empirically-based formula for \( s_{32} \) is:

\[
s_{32} \approx \frac{13}{n(n-1)} \sum (\ln L_i - \ln \bar{L}_{32})^2 \tag{2}
\]

where \( n \) is the sample size. Lauritzen (1982) states that the equation for \( s_{32} \) is approximate within 10-20%.

Some workers have used the arithmetic mean instead of \( L_{32} \) (Table 1). Sauter means are greater than arithmetic means because cubing large values effectively weights them heavier relative to lesser lengths, which was the intent of Blumberg & Curl (1974) and Curl (1974). Given the inverse relationship between scallop lengths and velocity, Sauter means yield lower velocities and discharges than arithmetic means. Blumberg & Curl (1974) directly measured velocities in their flumes, so their choice of the Sauter mean was presumably directly informed by their experiments and use of arithmetic means is not appropriate.

The arithmetic mean is itself skewed by extremely large and small values, but its use is most justified where data are symmetrically distributed around a central value such that largest and smallest values counterbalance one another. Hence, the arithmetic mean is commonly used for normally distributed values. Palmer (1976) justified use of the arithmetic mean on the basis that large scallops were the most complete and assumed that only the lengths of complete scallops were representative of formative velocities. Hence, she only measured scallops she regarded as complete, which she observed were most commonly the largest scallops. Palmer (1976) reports velocities calculated using the Sauter and arithmetic means. In all cases, as expected, the arithmetic means were smaller and velocities higher – often being twice that obtained from the Sauter mean. The effect of sampling bias is addressed below.

Hypothetically, if scallops formed only at a single velocity and were not modified between flows they would have a uniform distribution, but scallops downstream of joined passages equaled the sum of the discharges calculated for the individual passages. The total discharge was equivalent to the 85th to 95th percentile of flows measured at a spring (Lauritzen et al., 1985).
truncates one another as they migrate into and along walls and localized flow fields and substrate defects can create scallops across a range of sizes even when discharge is fixed (Villien et al., 2005). As a result, fields of scallops should be viewed as stochastic phenomena that – if we assume the hydrologic regime is not relevant to this study if we assume the shape of the statistical distribution of scallop lengths is unaffected by climate. 

CAVE HYDROLOGY

Scallop uncertainties

A total of 100 scallops were measured in adjacent "patches" with $n = 50$ for each sample. The two samples are combined because they are continuations of each

OBJECTIVES

This study explores the robustness of scallop-based velocities and discharges based on sample size. We explore common assumptions and other sources of uncertainty and potential error. Specific objectives include addressing basic questions, including those below:

- What are the statistical properties of the observed scallops?
- How confident can we be about velocities and discharges estimated from scallops?
- How is variability in inputs expressed in the final calculations?
- How will the results change if we change the sample size?
- What is the consequence of using the arithmetic mean instead of the Sauter mean?
- We explore these questions using field data, statistical tests, and Monte Carlo simulations.

METHODS

Scallop measurements in the 17-km long Boarhole-Portal Cave System in Greenbrier County, West Virginia, USA (38° N, 80.4° W). The temperate climate is typified by mid-latitude seasonality with precipitation falling year-round as both rain and snow. Annual average precipitation is 1,000 mm (Hardt et al., 2010). Floods are generated by a wide range of phenomena, including intense thunderstorms, snow melt, rain falling on snow, large frontal systems, and decaying tropical storms. Baseflow is highest in boreal late winter and early spring, but large floods can happen at any time of year. The scallops shown in Fig. 1 are a portion of 15-m long scalloped wall in an inactive elliptical tube. The tube is 4-m wide and 2-m tall and paleo-upstream evidence of paragenesis implies phreatic development. The elliptical tube is at the southwest entrance to a section called the K-surveys. The age of the passage is unknown and the scallops may have formed under a different climate regime than the one that prevails now. However, the hydrologic regime is not relevant to this study if we assume the shape of the statistical distribution of scallop lengths is unaffected by climate.
other. The scallops are within and adjacent to the area shown in Fig. 1. Scallops were measured without regard for their lengths or inferred “completeness”; choosing measurements based on completeness assumes all functional scallops have the same geometry, which may not be true, is inconsistent with Blumberg & Curl (1974), and introduces explicit user biases into the process. Lengths were measured to the nearest millimeter using a ruler with length defined as the distance separating the upstream and downstream scallop crests (Fig. 2).

Water velocities were calculated using Curl’s (1974) equations, which are valid for straight passage segments with parallel walls and elliptical cross sections. Each equation contains multiple constants, some of which are temperature dependent, and two unknowns: measures of scallop lengths ($L_{32}$) and passage dimensions (width or hydraulic diameter). The equation for an elliptical cross section is:

$$\bar{u} = \frac{\mu}{\rho L_{32}} Re^* \left[ 2.5 \left( \ln \frac{D_h}{L_{32}} - \frac{3}{2} \right) + B_L \right]$$

where $\bar{u}$ is mean velocity, $\mu$ is dynamic viscosity, $\rho$ is fluid density, $D_h$ is hydraulic diameter, and $B_L$ a constant related to velocity profiles. Based on experiments, $\mu$ and $B_L$ equal 2200 and 9.4, respectively (Blumberg & Curl, 1974). We used and values appropriate for water temperatures of 5ºC. $D_h$ is equal to $4\cdot$ (cross sectional area / length of the ellipse perimeter).

Data were processed and analyses performed using the free software R (R Core Team, 2019). As shown below, scallop lengths are log-normally distributed. The 100 Boarhole scallops failed a Wilks-Shapiro test for normality ($p << 0.001$), but their log10-transformed values yield $p = 0.06$ and their distribution is very similar to a normal distribution (Figs 3 and 4). However, the upper tail is heavier than the lower tail, may be the result of an unintentional sampling bias. We also measured 60 samples of scallops in active stream passages of three nearby caves. Sample sizes were from 30 to 40 scallops and 57 of the 60 sets are log-normal ($p > 0.05$) (Hall, 2019). But given the wide range in scallop lengths commonly observed in field data a large sample size is recommended to adequately test for log-normality because the Wilks-Shapiro test is sensitive to the tails of a distribution.

RESULTS

Statistical distribution

Lauritzen (1982) notes that scallops appear to be log-normally distributed. The 100 Boarhole scallops failed a Wilks-Shapiro test for normality ($p << 0.001$), but their log10-transformed values yield $p = 0.06$.

Fig. 3. A) Histogram of scallop lengths. Note the left skew; B) Histogram of log10-transformed scallop lengths (unitless). The distribution passes a Shapiro-Wilks test for normality ($p = 0.06$).

Fig. 4. Cumulative percent smaller diagram for all 100 scallops. The dashed line passing through the data represents a truly log-normal distribution, but the upper tail of the observed distribution deviates from the line and may be the reason the data marginally passed a normality test.
Sample sizes

Monte Carlo simulations were run in R using the 100 Boarhole scallops to obtain 10,000 Sauter means for each \( n \) in the range of 10 to 30 (Fig. 5). Discharges were calculated simultaneously by multiplying by passage area (Fig. 6). The Monte Carlo subsampling is equivalent to sampling the wall with no preference for scallop size or location, which is consistent with unbiased sampling techniques (citation). However, the large number of iterations per \( n \) and random chance led to extremes wherein subsamples were dominated by very small or very large scallops, which is a rough simulation of biased sampling. For small \( n \), subsamples dominated by large scallops yielded Sauter means outside the boxplot whiskers representing 1.5 times the interquartile range (IQR). The IQR is between the 25th and 75th percentiles. The outliers, as defined by R, are shown as circles (Fig. 5F). Larger sample sizes did not yield outliers when plotting boxplots.

As might be expected, the smallest subsample size \( (n = 10) \) yields a wide distribution (Fig. 5A), which is skewed to the right and ranges between 35 and 90 mm. These yield discharges \( (Q) \) between 3 and 9 m\(^2\) s\(^{-1}\) (3-9 cumecs) (Fig. 6A). For \( n = 10 \) to 17, the IQR extends outside the gray zone denoting \( L_{32} \pm 1 \) s.d. calculated using all 100 scallops. The IQR at \( n = 30 \) is well within the gray zone and the average Sauter mean (black bars within boxes) is close to the “true” value obtained from all 100 scallops (dashed line). The distribution of Sauter means narrows considerably between \( n = 10 \) and \( n = 30 \) (Fig. 5A-E), but the ends of the boxplot whiskers at \( n = 20 \) and \( n = 30 \) differ only by a few mm.

Discharge calculations are much more prone to outliers than Sauter means (Fig. 5F and 6F). At \( n = 30 \), discharges range from 3 to 8 cumecs, but the IQR is within the gray zone denoting \( Q \pm 1 \) s.d. calculated using all 100 scallops. All distributions (Fig. 6A-E) are skewed to the right, which shows that a subsample dominated by small scallops is more likely to yield outlier values than a subsample dominated by large scallops (Fig. 6F).

Choice of means

Using all 100 scallop measurements, the Sauter mean-based discharge is 5.1 cumecs and the arithmetic mean-based discharge is 6.7 cumecs (Fig. 7). We simulated the effect of mean choice when using \( n = 30 \) and generated 10,000 discharges using both \( L_{32} \) and the arithmetic mean. As would be expected, use of \( L_{32} \) yielded lower discharges than use of the
arithmetic mean (Fig. 7) because the Sauter mean places greater weight on large values and these yield lower velocities and discharges. There is comparatively little overlap in the $L_{32}$ and arithmetic mean-based distributions, demonstrating the non-trivial effect of which mean to use.

![Fig. 6. Distributions of discharges calculated using $L_{32}$ values obtained from the Monte Carlo simulations for different sample sizes. The range of possible values is still large even for $n = 30$, but the interquartile range of the simulated means falls within the shaded region in (F) corresponding to bounds created using the standard deviation of all 100 samples via equation (2).](image)

#### DISCUSSION

In theory, scallop length correlates to the water velocity associated with a dominant discharge (Lauritzen et al., 1985), but scallops on cave walls are rarely observed to be uniformly of one size because scalloping is a stochastic process influenced by many variables. Hence, the 100 observed scallop lengths vary by nearly an order of magnitude and are log-normally distributed, although the upper tail of the distribution marginally conforms to expectations based on the normal distribution (Fig.s 3 and 4). Based on field observations, the log-normality partially reflects truncation of some scallops by their neighbors, but presumably also local variations in flow and a host of other factors. After measuring ~1,800 scallops as part of a larger project, we note that many short scallops are complete and not the result of truncation. So, measuring only the largest “complete” scallops ignores the stochastic, non-linear phenomena responsible for scalloping. The stochasticism can be seen in the scallops reported by Blumberg & Curl (1974) and we recommend measuring scallops of all sizes, so as to

![Fig. 7. Density plots of discharges obtained from Monte Carlo simulation of sampling and calculations made with the Sauter mean ($L_{32}$) and arithmetic mean. The vertical lines represent discharges calculated using all 100 scallops and the associated choice of means.](image)
be consistent with the methods behind the formulas used in calculating water velocities and in recognition of the stochastic origin of scallops.

Arithmetic means calculated using some or all of the lengths will be smaller than equivalent Sauter mean-based values and bias velocities and discharges toward greater values (Fig. 7). The constants reported by Blumberg & Curl (1974) were back-calculated from known (measured) velocities and their calculations used the Sauter mean. Had they used the arithmetic mean, the estimated values of their “universal” constants would have been different. This and the choice of means may partially explain why others have reported different values for the constants reported by Blumberg & Curl (Goodchild & Ford, 1971). The origins of Re*, B*, equation (3) invalidates using the arithmetic mean in velocity and discharge calculations, but even setting that aside, randomly resampling 100 scallop lengths clearly demonstrates that using the arithmetic mean will bias discharges toward higher values. We recommend use of the Sauter mean because it is consistent with the methods used to obtain constants in the relevant equations.

The choice of sample size has significant impacts on the range of possible \( L_{32} \) values and hence precision. The fact that scallops are log-normally distributed means that small sample sizes may not adequately represent the full range of lengths at a given location and this is why the ranges of simulated \( L_{32} \) values in Fig. 5 become increasingly narrower with increasing sample size. Interestingly, the interquartile range of simulated \( L_{32} \) values for \( n = 20 \) is similar to \( \pm 1 \) standard deviation around the Sauter mean of all 100 scallops (Fig. 5F), which leads us to recommend sample sizes of no less than 20 scallops. Using a sample size of 30 will always be better than using a sample size of 20, but measuring those 10 additional scallop lengths will, on average, only marginally improve the precision of an \( L_{32} \) estimate (Fig. 5F).

The precision of velocity and discharge measurements improves significantly as \( n \) increases from 5 to 20 (Fig. 5A-C). For \( n \geq 20 \), The IQR of possible discharges is similar to \( \pm 1 \) standard deviation around the discharge calculated using the Sauter mean of all 100 scallops, but in this case the ranges represented by IQR and \( \pm 1 \) standard deviation are \( \sim 1 \) cumec.

This implies that the choice of which scallops to measure is significant enough that reported velocity and discharge values should at best extend to one or two significant digits. Reporting discharges similar to ours as precise three or more significant digits is overly optimistic and we encourage reporting them to only 1 or 2 significant digits. In our case, this would correspond to uncertainties of as much as 20%. Accepting this limited precision or large standard error is important if inferential statistics are to be applied to the velocities or discharges.

CONCLUSIONS

Scallops record flow conditions that might otherwise be unmeasurable and their lengths are inversely correlated with velocity. We find that a minimum of 20 to 30 scallop measurements should be made when studying them because their distributions are log-normal and smaller sample sizes yield poorly reproducible results. Scalars of all sizes should be measured in recognition of the stochastic origin of scallops and to avoid user biases. In fact, the choice of which scallops to measure can significantly affect the final outcomes of a study and the uncertainties are such that results should probably only be reported to one significant digit. Reporting greater precision masks the underlying uncertainties associated with the wide range of scallop sizes commonly observed and the possibility of sampling biases.

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Impacts of invasive rats on Hawaiian cave resources

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Abstract: Although there are no published studies and limited data documenting damage by rodents in Hawaiian caves, our incidental observations during more than 40 years of surveying caves indicate that introduced rodents, especially the roof rat, *Rattus rattus*, pose significant threats to vulnerable cave resources. Caves, with their nearly constant and predictable physical environment often house important natural and cultural features including biological, paleontological, geological, climatic, mineralogical, cultural, and archaeological resources. All four invasive rodents in Hawai‘i commonly nest in cave entrances and rock shelters, but only the roof rat (*Rattus rattus*) habitually enters caves and utilizes areas in total darkness. Skeletons and feces have been found in the deepest passages, sometimes over a kilometer from the nearest known entrance although the animals may have used nearby small, inconspicuous entrances. Their impacts include damage to rare native plants in cave entrances; predation on vulnerable cave-inhabiting species, such as *Thaumatogryllus* tree crickets, and native moths roosting in caves; destruction of the irreplaceable remains of the extinct terrestrial fauna; damage to organic material associated with cultural and archaeological resources, thereby obscuring the historical record of humans in the islands; introduction of unnatural nutrients into subterranean ecosystems via their bodies and feces allowing the colonization of caves by other harmful alien species; and disturbance of research sites. Furthermore, the extirpation of colonies of cave-roosting moths has impacted native birds nesting in the entrance and twilight zones.

Keywords: conservation, biospeleology, paleontology, archaeology, *Rattus rattus*

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INTRODUCTION

Caves, with their nearly constant and predictable physical environment, often protect important natural and cultural resources from degradation especially by weathering. In Hawai‘i, caves contain important biological, paleontological, geological, climatic, mineralogical, cultural, and archaeological resources (Howarth & Stone, 1982; Stone et al., 2007). Specifically, caves are home to diverse communities of highly specialized native animals adapted to live only underground (Howarth, 1991; Stone & Howarth 2007). Caves also hold an irreplaceable historical record of the extinct terrestrial biota of the islands (James & Olson, 1991; Burney et al., 2001; Paulay & Starmer, 2011; Ziegler et al., 2016) as well as a record of human culture through time (Sinoto, 1992; Bolt, 2005; Burney & Kikuchi, 2006). In addition, cave entrances often provide refuge for unique native plants and animals (DLNR, 2003). There are no native rodents in Hawai‘i, but four species have been introduced to the islands by humans: the Polynesian rat (*Rattus exulans*) was introduced to Hawai‘i circa 800 years ago by the early Polynesian voyagers; the house mouse (*Mus musculus*) and brown rat (*Rattus norvegicus*) were introduced post 1778; i.e., shortly after western contact; and roof rat (*Rattus rattus*) established in the 1870s (Atkinson, 1977; Tomich,1986). All four species commonly nest in cave entrances and cavities, but only the roof rat (*Rattus rattus*) habitually enters caves and can utilize areas in total darkness. *Rattus exulans* prefers open habitats in the lowlands, but it is widely distributed in the islands and also occurs in upland rain forests. It can climb trees to feed but nests on the ground. *Rattus norvegicus* prefers open habitats in the lowlands, but it is widely distributed in the islands and also occurs in upland rain forests. It can climb trees to feed but nests on the ground. *Rattus norvegicus* prefers urban and lowland open habitats. It nests on the ground and rarely climbs trees. It is rare or non-existent in upland rain forests. *Rattus rattus* is the most plastic in the habitats used by the rodents in Hawai‘i and is the dominant rat in many
habitats. It is an excellent climber and nests both on the ground and in trees (Atkinson, 1977; Stone et al., 1984; Tomich, 1986).

Although there are no published studies and limited data illustrating damage to cave resources by non-native rats in Hawai‘i, our incidental observations during more than 40 years of investigating caves indicate that introduced rodents, especially the roof rat, Rattus rattus, poses significant threats to vulnerable cave resources. Our goal here is to describe the environmental impacts of non-native rats in Hawaiian caves, including damage to rare native plants in cave entrances, predation on biological resources, destruction of the irreplaceable remains of the extinct terrestrial fauna, loss of organic material associated with cultural and archaeological resources, introduction of unnatural nutrients into subterranean ecosystems, and disturbance of research sites.

METHODS

We examined as many caves as possible under the restraints placed by the objectives that were defined under each grant or contract. Our cave surveys were conducted between 1971 and 2012 and included limestone and lava tube caves on Kaua‘i and O‘ahu, lava tube and piping caves on Moloka‘i, and lava tube caves on Maui and Hawai‘i islands. On Hawai‘i island, the lava tubes surveyed ranged between sea level to 3,900 m elevation above sea level. Lava tubes are common landforms on young shield volcanoes, and range in size from a few meter-long shelters to extensive systems 50 km or more long and descend over 1,000 meters in elevation. (Kauahikaua et al., 2009; Richards et al., 2018).

The caves were explored by teams using standard caving and safety techniques (NSS, 2019; Wynne et al., 2019). Our research focused primarily on cave-inhabiting arthropods and their adaptations to the environment (e.g., Hoch & Howarth, 1999; Stone & Howarth, 2007; Wessel et al., 2013); however, the presence and condition of other resources were noted when encountered. A few caves were entered repeatedly and studied intensively for ecological research (e.g., Howarth, 1981; Howarth, 1983). We also participated in cave projects that focused on paleontology (e.g., James & Olson, 1991; Ziegler et al., 2016), archaeology (e.g., Hammatt et al., 1978; McEldowney & Stone, 1991; Howarth & Stone, 1993), and management (e.g., Stone et al., 2007).

Rats and their impacts were not the primary focus of our cave inventories, but evidence was noted when recognized. Thus, our observations likely underestimate the level of damage since more severe damage was more likely to be recognized than would more cryptic impacts. Since rat occurrence in caves was not the focus of our work and preservation was often poor, voucher specimens of the bones of dead rats were not collected.

RESULTS AND DISCUSSION

During our early surveys, we could not determine whether the occasional rat skeleton found represented animals living in the caves or were accidentally in caves and became lost as victims of their wanderlust behavior. However, the frequency, types and location of rat damage observed indicated that rats were frequently entering caves for shelter, food, and water. Also, rats often found and disturbed our bait stations and pitfall traps relatively quickly, often within a day or two of their deployment, which further corroborated their ability to navigate and exploit resources in complete darkness. Importantly, the presence of fresh rat feces at the disturbed study sites confirmed that the culprits were rats.

Rodent remains found in deep cave zones were apparently all Rattus rattus. Species determination was confirmed for rats captured in snap traps set to protect our traps and baits used to sample cave animals. For other carcasses, determination was based on size of the remaining skeleton. R. exulans is significantly smaller than R. rattus; whereas R. norvegicus is usually considerably larger (Tomich, 1986). In addition, R. rattus is an excellent climber and occurs in a wider range of habitats than the other species of rats (Atkinson, 1977; Stone et al., 1984) and, therefore, is more likely to be found in caves.

Evidence of the presence of rats was noted in at least a few caves on all islands, but most observations of rat damage to cave resources were made in lava tube caves on Maui and Hawai‘i islands. Specifically, rat damage was noted in five lava tube systems on Haleakala on Maui, and in many of the 100s of lava tube systems surveyed on Kīlauea, Mauna Loa, and Hualalai volcanoes on Hawai‘i island. Although we collected data on rat presence or damage in a non-systematic way, a survey of 14 lava tubes within Hawaii Volcanoes National Park (Howarth & Stone, 2008) provides a rough approximation of the scope of the problem. The caves ranged from near sea level to 3,800 m in elevation. Rats and rat damage were recorded in nine caves from sea level to 1,525 m, i.e., 64% of the total caves surveyed.

Resource damage by rats was observed from the entrance to deep within caves, sometimes more than a kilometer from the nearest known entrance -- although rats would be able to use small inconspicuous entrances to gain access to these areas of the caves. The degree of damage ranged from incidental feeding to severe; that is, nearly complete destruction of the resource. For example, rat damage was noted on a wide range of cave resources including feeding on rare native plants growing in the entrance zone, preying on native cave-inhabiting arthropods, damaging archaeological and cultural materials, gnaw marks on or completely destroying paleontological deposits, and disturbing research sites. In addition, dead rats and their feces provide novel food, which may allow other non-native species to colonize the caves. These impacts are described in more detail below.

Damage to rare native plants in cave entrances

Pit-like cave entrances often provide refuge habitats for native plants since pits capture and retain moisture and nutrients beneficial to plant growth (Howarth, 1983). Equally important, pits prevent alien ungulates from entering and eating the plants (Wynne et al.,
Many rare and endangered native Hawaiian plants survive in these refuge pits (USFWS, 1996; DLNR, 2003). Rodents, however, can easily enter pits and seriously damage vulnerable species. Impacts include damage by their chewing on plants (Fig. 1) and by disrupting successful reproduction especially since seeds and fruits would tend to remain within the pit (Chimera & Drake, 2011). Several federally protected native plant species are known primarily from cave entrance pits on Hawai‘i island: notably Delissea undulata (Campanulaceae) and Pleomele hawaiiensis (Asparagaceae) (USFWS, 1996; Richards et al., 2018). Rat depredation is considered a serious threat to native Hawaiian plants (USFWS, 1996). Stone (1985) lists examples of endangered plant species damaged by Rattus rattus in Hawai‘i.

**Damage to biological resources in caves**

Rats are omnivores and will feed on almost any organic material they find. Their scavenging on decaying organic matter may compete with native scavengers. They feed on plant roots in caves, and thus, compete with native rhizophagous arthropods, and they prey on any cave animals they encounter. Cave-adapted arthropods may be especially vulnerable since they generally move slowly even when disturbed. For example, *Thaumatogryllus* (Fig. 2) cave crickets, found on Hawai‘i and Maui, are large (>1 cm in length) and feign death when disturbed. This behavior would make them especially vulnerable to rat predation.

Rats are implicated in the extinction of native cave species, especially several species of large moths that once roosted in Hawaiian caves in huge colonies (Ziegler et al., 2016) much like bats and the continental cave crickets do in other regions. Two groups of native moths displayed this behavior: an underwing *Hypocala velens* in the family Erebidae, and cutworms in the genus *Peridroma* (and perhaps also the related *Agrotis*) in the family Noctuidae. Perkins (1913) reported that *H. velens* adults roosted in caves and rock crevices and emerged at dusk in huge numbers. Perkins found these colonies were more common in the lowlands, but he discovered a large colony in a cave on Haleakalā, Maui, at 2,750 m elevation. We found only one cave specimen of this species during our surveys; the adult moth was roosting on the ceiling in a cave on the north slope of Mauna Loa at approximately 1,500 m. Although now rare, the species may persist undetected in small colonies.

There are about six *Peridroma* species endemic to Hawai‘i and 16 species of the closely related *Agrotis*. Several species of *Peridroma* once roosted in caves, and a few colonies still survive in high-elevation caves on Mauna Loa (Bonaccorso et al., 2016; Ziegler, 2016). The cold, dry rocky desert over the caves is largely inhospitable to rodents and probably has impeded their invasion. In the 1980s, we found a large colony, numbering many 1000s of individuals, of an unidentified *Peridroma* species roosting in a cave on the north slope of Mauna Loa at about 3,900 m. Moths leaving the cave at dusk formed a huge funnel cloud darkening the sky for 10 to 15 minutes as the moths dispersed downslope towards the saddle between Mauna Loa and Mauna Kea. This colony disappeared circa 1990. In September 2005, we found a second colony of *Peridroma* in a nearby cave at 3,800 m. This colony numbered at least a few hundred adults, but an accurate count was not possible because moths were tightly clustered in crevices on the walls and ceiling (Fig. 3). A permanent pool of ice covered the floors of both caves. How cave roosting moths find their way back to their roost is unknown but may be analogous to the cave roosting Bogong moth, *Agrotis infusa*, in Australia (Warrant et al., 2016).

A few other colonies of moths in caves on Mauna Loa have been reported in the literature. Bunnell & Giffin (2000) collected a dead *Peridroma albiorsis* in Big Red Cave, a lava tube at 3,000 m elevation on Mauna Loa. Bonaccorso et al. (2016) reported living and dead adult moths (*Peridroma*) in 13 lava tube caves between 2,200 and 3,600 m elevation on Mauna Loa. Incidentally, the native Hawaiian bat, *Lasiusurus cinereus semotus*, was detected hunting around the cave entrances at dusk (Bonaccorso et al., 2016).

In addition to bats, native birds were also associated with these moth colonies. The ‘Apapane (*Himatione...*
sanguinea) frequently nested in the entrance and twilight zones of caves that supported moth colonies. Van Riper (1973) reported ‘Apapane nesting in several high-elevation caves on Mauna Loa and Hualālai but did not note the association with moth roosts. We found dead moth remains in all the caves in which we found ‘Apapane nests (Fig. 4). Finding ‘Apapane nests in caves is highly unusual as this species typically nests high above the ground in trees (Van Riper, 1973). However, the easy access to adult moths as food for nestlings would have provided a strong stimulus for this behavior. Many of these nest sites in caves are no longer active indicating that the loss of moth colonies may have also decreased the distribution and abundance of ‘Apapane. In addition, the nests accessible to rats would be vulnerable to predation.

Cave-roosting moths probably occurred on all the main islands, but except for Perkins’ (1913) observations on Hypocela on Maui and lowlands generally, records for islands other than Hawai‘i and Maui are lacking. Cave-roosting moths may have been an important faunal element in Maui caves in the recent past, but there is no evidence that any survive. A large colony of an unidentified Peridroma species once occupied Crystal Cave in Haleakalā Crater at 2,300 m elevation. The entrance is wide and low and leads to a single room 10-15 m in diameter, which is all in twilight. At the time of our survey in 1976, the dry floor was entirely covered with a several centimeter-deep layer of dust and moth fragments. No living moths were found. Rat feces were also abundant, which suggested a reason for the collapse of this colony of moths. However, populations of native cutworms have also sharply declined early to mid-20th century due to the purposeful introduction of biological control agents to control their larvae (Gagné & Howarth, 1985). The larvae of a few of these native cutworms became pests of lawn grasses, crops, and other valuable plants, and several non-native predators and parasites were introduced in an attempt to control these pest species (Gagné & Howarth, 1985). Thus, it may have been a combination of introduced biological control agents and rats that resulted in the substantial loss of cave-roosting moth populations.

Damage to paleontological resources in caves
Caves are the premier depositories of the remains of pre-human life in the islands (Burney et al., 2001). Cave entrances often act as pitfall traps for ground dwelling animals (James & Olson, 1991).

Plants that grow in or near entrances can become buried by sediments falling into pits during floods or collapse of the pit walls (Burney et al., 2001). Surface-inhabiting animals occasionally enter caves, and some become disoriented and die there. Their remains provide a discontinuous record of island life over time (James & Olson, 1991; Olson & James, 1991; Paulay & Starmer, 2011; Ziegler et al., 2016). So far, buried fossils are protected from direct rat damage (e.g., Burney et al., 2001), but organic and mineralized fossils exposed on the cave floor can be damaged or destroyed by rats (Fig 5). Rats may be rapidly gnawing their way through this unique record of the history of life in the islands. We have noted substantial rat damage on fossils of extinct land snails, crabs, and birds (Fig. 6).

Damage to cultural and archaeological material in caves
During pre-contact times, i.e., between the arrival of Polynesians circa AD 1200 (Wilmshurst et al., 2011) and the arrival of Europeans in 1778, the native Hawaiian peoples used caves extensively for habitation, refuge, water catchment, burials, caches, and many cultural and ceremonial purposes (Martin, 1992; Sinoto, 1992;
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Bollt, 2005; Esh et al., 2008). Organic and mineral artifacts left in caves by Hawaiians are susceptible to the depredations by rats. Once destroyed the evidence of human use of caves is lost, and the historical record of humans in the islands is obscured. For example, Hawaiians commonly excavated small water catchment basins into the cave floor beneath drips (Martin, 1992) (Fig 7). Cultural material left at these basins reveal how and when they were made and used (Esh et al., 2008). Rats can damage or destroy much of this evidence thereby erasing part of the cultural history of Hawai'i. Rat damage at water catchment sites includes destruction of marine shells used as dippers, remains of torches and other organic debris associated with the basin, and calabash gourds (Fig. 8) used to collect and transport water.

**Introduction of unnatural nutrients into caves**

The roof rat (R. rattus) is pervasively invading into the deepest parts of caves in search of food, water, or from wanderlust. Feces (Fig. 9) and diaspora carried into caves by rats, along with their dead bodies, introduce unnatural foods into the subterranean ecosystem that may allow other harmful alien species to colonize caves. Populations of non-native cavernicoles that may benefit from the presence of rats include a few species each of isopods, springtails, beetles, and flies. Among the flies, non-native scuttle flies (Phoridae) commonly are attracted to rotting meat and thus suspected of feeding on rat carcasses; however, these small flies rarely leave any trace on skeletons.

Many (uncounted but perhaps 5%) rat skeletons in caves are associated with large accumulations of calyptrate fly puparia. Curiously, all that have been identified belong to the muscid fly, Synthesiomyia nudiseta. Apparently, S. nudiseta can out-compete the other approximately 15 species of large non-native necrophagous flies recorded in Hawai'i, and complete development in total darkness. In arthropod surveys in surface habitats, S. nudiseta is often present but is not as common as many related species (Howarth et al., 2012).

A few native cave species, such as some collembola, isopods, millipedes, and flies, can also feed on this imported material. Steffan (1973) described a native black fungus gnat, Phytosciara (Prosisciara) vulcanata, from adults reared from fungus covered rat feces.

**Disturbance of research sites**

The speed and frequency at which rats find and disturb bait stations and pitfall traps in caves indicate that they are frequent visitors and well-adapted to navigate complex mazes in total darkness. Rats also occasionally disturb or damage study
sites and equipment such as atmometers and hygrothermographs. Their presence has required us to protect sampling stations from their depredations by placing sampling material on high ledges or under rocks; that is, out of reach of rats. When possible, we also set snap traps, which both lowers the risk they represent, while also providing additional bait for our sampling (Fig. 10). We have not yet found evidence of rats nesting in deep caves and conclude that rats probably are not residents within caves but visitors.

Conservation implications

We have documented examples of real and potential threats caused by invasive rats on cave resources in Hawai‘i. However, since our observations were incidental and not quantified, the true severity of these threats is unknown. We, therefore, encourage researchers and other persons studying caves to be aware of the problem and accurately record evidence of rat activity and related damage, not only in Hawai‘i but wherever invasive rats occur. With better documentation, it will be possible to perform specific research programs to quantify rat impacts and to develop appropriate monitoring and mitigation programs. Several public and private agencies in Hawai‘i are supporting research on protocols to effectively control rats in sensitive habitats [e.g., Department of Land and Natural Resources (DLNR), National Park Service, and The Nature Conservancy]. Caves should be included in that effort.

In a few instances where we noted rat damage was severe and on-going, we made recommendations to control rat populations in and around the affected cave or to fully inventory and/or retrieve the resource. However, many of the examples we recorded are so widely scattered in different caves, that, with few exceptions, it is not feasible at present to develop programs to address the issue.

One issue that should be addressed currently is the impact of rats on populations of cave-roosting moths. Currently, most large colonies of these moths occur outside the range of invasive rodents; that is, in lava tubes on sparsely vegetated lava flows at high elevation. The cold environment with few food resources for rodents has slowed their dispersal into this habitat. However, they would be able to follow human hiking trails and roads, especially if humans are discarding trash along the trail. Trail mixes of grains, nuts, and dried fruits are excellent baits for rats. High altitude trails should be monitored for the presence of rats and the discarded organic material removed. In the early 1990s, resource managers at Hawaii Volcanoes National Park rerouted a summit trail on Mauna Loa to bypass a cave with a known population of moths in hopes of preventing rats from finding the cave. Additionally, caves harboring moth colonies or nesting birds should be monitored regularly, and if rats are suspected, an appropriate control program should be initiated before the colony collapses.

Problems posed by rodents outside of Hawai‘i were beyond the scope of the present study although such impacts certainly occur. *Rattus rattus* is a cosmopolitan pest widely recognized as one of the most damaging invasive species worldwide (Global Invasive Species Database, 2020). Our observations in Hawaiian caves indicate that rats pose similar problems in other regions of the world. We encourage researchers to record their observations of rodent activity in caves. Caves in continental areas often harbor native vertebrates, including rodents [e.g., *Neotoma* rats in North American caves (Dunning & Payne, 1979; Clark et al., 1994)], which may also disturb cave resources. For example, Clark et al. (1994) provide circumstantial evidence of the native wood rat, *Neotoma floridana* preying on endangered Ozark big-eared bats (*Corynorhinus townsendii ingens*) roosting in Oklahoma caves.
ACKNOWLEDGMENTS

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INTRODUCTION

Microbial communities are able to exist and thrive in a multitude of environments, including those that are harsher habitats such as caves that were previously thought to be completely devoid of life (Coughlan et al., 2015). Challenging characteristics of some cave habitats include the lack of light, low and limited amounts of organic nutrients, higher humidity and higher concentration of minerals that are normally natural DNA blocking agents. These demanding features contribute to distinctive cave environments (Cheeptham et al., 2013; Ghosh et al., 2016), and each cave has unique characteristics. Hence, cave microorganisms must adapt to possess some specialized metabolic pathways to enable them to survive and thrive within them (Ghosh et al., 2016). The broad diversity of microorganisms within caves encompass members among Proteobacteria, Firmicutes and Actinobacteria.
In particular, cave sediments house interesting microbial communities that include species of Streptomyces, Arthrobacter, Leifsonia, Rhodococcus, Bacillus, Paenibacillus, Pseudomonas, Williamsia, Leifsonia, Nocardia, Devosia (Ghosh et al., 2016; 2017), and interactions due to the unique conditions of the cave environment (Barron et al., 2010). These bacterial communities exist within caves are able to acquire their energy in a number of ways, that comprises breaking down complex aromatic compounds, fixing volatile organics as well as carbon dioxide and nitrogen from the atmosphere, and oxidizing reduced metals within rocks (Barton & Jurado, 2007; Ghosh et al., 2016). Due to the fact that there is limited and chemically complex nutrients within a cave system, very few microbial species are able to uptake and perform catabolic reactions for growth (Barton & Jurado, 2007). Thus, many microbial communities work in a mutualistic cooperative relationship to overcome this nutrient limitation (Barton & Jurado, 2007). The unique cave environment that possesses a myriad of bacterial communities can be utilized for the potential discovery of new antibiotics (Ghosh et al., 2016; Gosse et al., 2019).

The need for discovery of new antibiotics is on the rise, since pathogens continuously becoming resistant to antibiotics, and those currently available either not having specific inhibitory activities or pose concerned side effects (Cheeptham et al., 2013; Ghosh et al., 2016). In terms of the cave microbiome, a number of studies have been explored for its diversity and has exhibited a wide range of antimicrobial properties (Cheeptham, et al., 2013; Ghosh et al., 2017; Gosse et al., 2019). For instance, in a recent study from Iron Curtain Cave, Canada, two bacterial isolates were identified ICC1 and ICC4, that exhibited antagonistic properties against the multidrug resistant E. coli strains (Ghosh et al., 2017). Further genomic and metabolome analysis revealed that though the isolates are highly homologous with the terrestrial bacteria Streptomyces lavendulae but there were not the same, and possess expanded metabolic potentials (Gosse et al., 2019). These results have extended potential scopes from the caves and more extreme environments to be an effective resource for novel biologics and antibiotics (Ghosh et al., 2016).

In this study, we investigated the diversity of cultivated bacteria from seven different locations within the Raspberry Rising Cave, Canada, which has never been reported earlier. The phylogenetic analysis of 16S rRNA gene sequences of the cultivable bacteria were performed and furthermore, antimicrobial properties of these cultivated bacteria against the multidrug resistant bacterial strains were assessed. The Tupper-Raspberry cave system has fairly demonstrated us that it could be a potential source for some crucial microbes, such as phyla belonging to Actinomycetes, Proteobacteria, Firmicutes, Bacteroidetes, and novel biomolecules.

**MATERIALS AND METHODS**

**Cave description: The Tupper-Raspberry cave system**

In Glacier National Park of Canada, a large sink takes meltwater from the Tupper Glacier. Known as Tupper Sink, water entering it was dye-traced by Ford and others from the Karst Research Group at McMaster University (Ford, 1967). The waters were positively traced to a spring 483 m lower and 1940 m distant. This spring, known as Cascade Cave, or Raspberry Rising, has been known for over a hundred years. This spring had been explored by cavers and open cave passages characterized by a flowing streamway were explored for 70 m to a sump. The sump was first passed by cave diving methods in 1972, where the passage rose from the water and emerged into an air-filled room with the water flow emanating from a high waterfall (Rollins, 2004). This waterfall obstacle remained unpassed until modern explorations began in 2012 (Graham & Stenner, 2019).

The system is obstructed by water at both ends, by the sump 70 m after the entrance and by the sink at the top taking the meltwater from the Tupper Glacier. Exploration of the cave is limited to the winter months, due to dangerous flow rates which prevent diving through the sump and contribute to high water levels within the remainder of the cave. In between the sump and the Tupper Sink, an open, vadose cave system is present. Once past the waterfall, a mixture of open cave passages and large rooms have been explored, in a cave system with multiple levels. Five flooded sumps have been discovered and explored, so far, and the cave system has proven to be remarkable due to the quantity and quality of unique speleothems (Graham & Stenner, 2019). The cave system is confined to a 20-60 m wide marble band sandwiched between calcareous slates and garnet schist identified this system as a classic Type 1 stripe-karst hydrology (Yonge, 2013).

The system has now been explored to 5,495 m in length and a vertical range of 219 m. As of January 2019, it is the longest marble cave system in Canada, the tenth longest and 26th deepest overall cave in Canada and is the second longest cave in all of Canada’s National Parks (Graham & Stenner, 2019).

The system had only been explored to the waterfall immediately after the sump, resulting in 76 m of surveyed cave passage (Rollins, 2004). The remainder of the cave passages, having only been explored since 2012, had been devoid of human contact. The majority of passages, outside of the main route within the cave, were only visited during initial surveying and have not otherwise seen human traffic. This represents a unique opportunity for cave sediments and speleothem sample collections in a cave system while it underwent initial exploration.

The Tupper cave system is designated as an Environmentally-Sensitive Site within Parks Canada Zone 2 Wilderness. Entry into an Environmentally-Sensitive Site requires the highest level of care in order to protect sensitive geological resources and ensure minimal intervention in ecological processes.
Exploration and research in Raspberry Rising were made possible by Parks Canada via “Tupper Cave System (Tupper Sink/Raspberry Rising) Exploration” Research and Collection Permit GLA-2016-23196.

**Sample sites and site selection**

Sample sites within the cave were chosen for their diversity and consisted of locations within the cave that had distinctly different features from one another. These sites were of varying distances from the entrance, cave sediment types, and speleothem types. Cave sediment samples were collected aseptically from seven different sampling areas from locations in the cave beyond the initial sump 70 m from the entrance (Fig. 1). The average cave sediment temperatures measured was 4.8°C.

![Map of Raspberry Rising Cave](image)

**Samples 1 and 2**

During the exploration of the cave on 9 April 2017, a squeeze between breakdown rocks revealed a small unexplored room connecting two known passages approximately 750 m from the entrance. This room was home to a sloping floor of loose sediment. With this opportunity to collect cave sediments which unequivocally had not been disturbed by humans at this time samples were collected of both top cave sediments and cave sediments from a dig of one-ft depth (Fig. 1) ([Supplementary Table 1](#)).

**Samples 3 and 4**

These samples of cave sediments were located in passage characterized by an active stream flow 123 m from the entrance. The cave sediments were in a sandbar like deposit to the side of the flowing water. This sandbar is expected to have increased water saturation or even could be submerged every summer due to increased flow rates from the meltwater of the Tupper Glacier entering the sink. Top cave sediments and cave sediments from ½ ft deep were separately sampled, deeper cave sediments were unobtainable due to the dig hitting a layer of bedrock (Fig. 1) ([Supplementary Table 1](#)).

**Sample 5, 6, and 7**

Samples 5, 6, and 7 were all co-located in a rift passage, high on a ledge approximately 4 meters above the active stream way and 119 m from the entrance. This area is expected to remain dry during summer floods. On the wall of the ledge a patch of multi-colored loose flaky material approximately 50 cm by 30 cm was located. This material was scraped from the wall using sterile instruments and captured as Sample 5. Sample 7 was from the same patch of material on the wall but a specifically loose and powdery section that was all orange colored. Sample 6 consisted of scrapings, in the form of powder, from a vein of orange material on the ceiling close to the wall mat (Fig. 1) ([Supplementary Table 1](#)).

**Sample collection and bacterial isolation**

Approximately 10 g of cave sediment samples (Sample #1-4) and cave wall scrapings (Sample #5-7) (Fig. 2) were placed in sterile 250 mL volumetric flask. The samples were rinsed with sterile deionized water on a shaking incubator at 8°C, 150 rpm overnight. One hundred microliter of the undiluted and decimally diluted (10⁰, 10⁻¹, 10⁻², 10⁻³) cave sediment washings were plated on the three different media plates, namely R2A (Teknova, Hollister, CA, USA), Hickey-Tresner (HT) (Yeast extract 0.1%, Beef extract 0.1%, 0.2%, Dextrin 1%, pH 7.3) ([Cheeptham et al., 2013](#) (Campbell Company, Toronto, ON, Canada), and Difco™ Actinomycetes Isolation (AI) Agar media (Thermo Fisher Scientific Inc., Waltham, MA, USA). The plates were incubated at 8°C till 7–8 weeks/until the visible colonies were observed. Morphologically distinguishable colonies were selected and re-streaked.
Fig. 2. Raspberry Rising Cave, Canada. A) Sampling site ≈750 m from the entrance; B) Sampling site 123 m from the entrance; C), D), E), F), G), H), I), J), K) and L) are the sampling sites 119 m from the entrance; The red, yellow and white arrow head indicates the cave sediments, yellow microbial mats and sampled cave wall scrapings respectively.

on the same media plates to obtain pure cultures. Each of the pure bacterial isolates were further inoculated in 3 mL volume of the respective broth media (R2A, HT, AI), incubated for a period of 1-2 weeks, followed by bacterial genomic DNA extraction. An aliquot of the grown cultures was stored in 20% (v/v) glycerol at -80°C.

MOLECULAR TAXONOMY

Genomic DNA extraction and sequencing

Genomic DNA (gDNA) extraction from the bacterial isolates were performed as per previous studies (Hoffman & Winston, 1987; Ghosh et al., 2017). Following the extraction, the gDNA was subjected to
polymerized chain reaction amplification of the 16S rRNA gene as described previously (Ghosh et al., 2017). The 27F (5'-AGAGTTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') primers were used for the amplification reaction. The PCR amplicons obtained were further resolved on an Ethidium bromide –Agarose (0.8%) gel in order to confirm their amplification. The unpurified amplicons were sent to Macrogen, Seoul, South Korea for nucleotide sequencing. The DNA sequences obtained were analyzed using the BLAST algorithm with the available sequences in the GenBank at the National Center for Biotechnology Information (NCBI) http://www.ncbi.nlm.nih.gov/genebank/index.html (Altschul, et al., 1997). 16S rRNA gene sequences were identified with the >98% identity and >80% coverage to the closest homologue in the GenBank and DNA Data Bank of Japan and were assigned with gene accession numbers (Supplementary_Table_3). The sequences that were not allotted with the GenBank or DDBJ number will be considered for further analysis.

**Molecular Phylogenetic analysis**

The evolutionary history was inferred using the Neighbor-Joining method (Saitou & Nei, 1987). The optimal tree with the sum of branch length = 2.09133087 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al., 2004) and are in the units of the number of base substitutions per site. The analysis involved 103 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There was a total of 250 positions in the final dataset. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

**Antimicrobial activity determination**

The cave bacterial isolates were subjected for antimicrobial activities screening against the multidrug resistant (MDR) and regular non-resistant bacterial strains. The MDR *E. coli* (New Delhi strain) #15-318, *E. coli* #15-102 and methicillin-resistant *Staphylococcus aureus* (MRSA)-43300 while the regular non-resistant strains *E. coli*-33 and *S. aureus* were chosen. All the test strains used were procured from our previous studies (Ghosh et al., 2017; Ghosh et al., 2018). The bacterial strains were inoculated in 3 mL of the nutrient broth (Criterion™Dehydrated CultureMedia, Hardy Diagnostics, CA, USA) and incubated overnight on a shaking test tube rotator overnight at 37°C. The antimicrobial activities assay was conducted employing seeded agar method. All the tested bacterial strains were inoculated at a concentration of 10^6 cfu/mL in 250 mL molten agar media with a gentle shaking and poured in a Nunc® Bioassay Dish (245 mm x 245 mm x 25 mm) (Cole-Parmer Scientific Experts, Montreal, QC, Canada). Two screening techniques, the plug and the toothpick assays, were adopted to screen the cave bacterial isolates against the tested bacterial strains. The former technique involved aseptically cutting of the agar plugs (0.5 square centimeter) using sterile scalpel from each of the cave bacterial culture plate, while the latter include aseptically picking up of the cave bacterial colony using sterile tooth picks. Both agar plugs and the bacterial isolate were finally placed on to the tested microbe’s-seeded agar plates. The antimicrobial assay plates were prepared in duplicates and incubated at 8°C for a period of 2-3 days. The antimicrobial activities were determined as the zone of inhibition around each bacterial colony. The diameter of the zone of the inhibition was measured manually with electronic Vernier caliper (Guilin, Guangxi, China).

**RESULTS**

**Bacterial diversity**

A total of 103 bacteria, based on their colony characteristics on the specific culture growth media, were isolated from cave sediments and wall scrapping samples of the Raspberry Rising Cave. A set of 34 bacteria each were isolated on isolated on R2A- and HT- agar media while 35 bacteria were cultivated on Al-agar (Table 1).

Genomic DNA extraction were performed from each the cultivated bacterial isolates followed by the 16S rRNA gene amplification. The PCR amplification revealed that the amplicons ranging from 1300-1500 bp. Based on the nucleotide sequencing of each of the rDNA amplicons, the bacterial isolates were categorized into three major phyla of Proteobacteria, Actinobacteria and Bacteroidetes. Further investigations, revealed the genera of *Pseudomonas* (48.54%) exhibited the majority of the population followed by *Rhodococcus* (39.80%) and *Flavobacterium* (3.88%). The genus *Janthinobacterium* and *Arthrobacter* contributed about 2.91% each, of the total population while, 0.99% bacterial population were recognized as endophytic *Proteobacteria* (Fig. 3) (Supplementary_Table_2, Supplementary_Table_3).

Noteworthy, 5.82% of the bacterial population exhibited ≤ 98% when compared to the available 16S rRNA gene sequences and could not be assigned with the Gene Accession numbers neither from the GenBank nor from the DNA Data Bank of Japan (DDBJ) (Supplementary Table_2, Supplementary Table_3).

The *Pseudomonas* and *Rhodococcus* were found to be widely distributed in all the sampling points that includes sampling points 1,2,3,4,5 and 7, 2,3,4,5,6,7 respectively while *Flavobacterium* and *Janthinobacterium luidum* were distributed at two and three sampling points respectively. *Paeniglutamicibacter* sp. and *Arthrobacter* sp. were scarcely distributed and only observed at a single sampling point (Fig. 3).

**Phylogenetic analysis**

Dendogram deduced from the 16S rRNA gene sequences of these bacteria revealed that *Proteobacteria*...
Table 1. Sampling locations with their respective characteristics in the Raspberry Rising Cave (RRC). Also, denoting the isolation media used to cultivate the bacterial isolates.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Sampling locations</th>
<th>Location characteristics</th>
<th>Isolation culture media</th>
<th>Bacterial isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Wall of No Return</td>
<td>Cave sediments 1FT depth</td>
<td>R2A</td>
<td>RRC1-RRC5</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HT</td>
<td>RRC6-RRC7</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Act</td>
<td>RRC8-RRC14</td>
</tr>
<tr>
<td>2</td>
<td>Wall of No Return</td>
<td>Top Cave sediments</td>
<td>R2A</td>
<td>RRC15-RRC20</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HT</td>
<td>RRC21-RRC25</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Act</td>
<td>RRC26-RRC29</td>
</tr>
<tr>
<td>3</td>
<td>Station 44</td>
<td>Top Cave sediments</td>
<td>R2A</td>
<td>RRC30-RRC34</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HT</td>
<td>RRC35-RRC39</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Act</td>
<td>RRC40-RRC45</td>
</tr>
<tr>
<td>4</td>
<td>Station 44</td>
<td>Cave sediments 1/2FT depth</td>
<td>R2A</td>
<td>RRC46-RRC49</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HT</td>
<td>RRC50-RRC54</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Act</td>
<td>RRC55-RRC59</td>
</tr>
<tr>
<td>5</td>
<td>Station 17</td>
<td>Wall Scraping</td>
<td>R2A</td>
<td>RRC66-RRC71</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HT</td>
<td>RRC61-RRC65</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Act</td>
<td>RR72-RR76</td>
</tr>
<tr>
<td>6</td>
<td>Station 17</td>
<td>Wall Scraping</td>
<td>R2A</td>
<td>RRC77-RRC80</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HT</td>
<td>RRC81-RRC85</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Act</td>
<td>RRC86-RRC88</td>
</tr>
<tr>
<td>7</td>
<td>Station 17</td>
<td>Wall Scraping</td>
<td>R2A</td>
<td>RRC89-RRC92</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>HT</td>
<td>RRC93-RRC98</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>Act</td>
<td>RRC99-RRC103</td>
</tr>
</tbody>
</table>

(51.45%), were the major phylum followed by Actinobacteria (43.68%) and Bacteroidetes (3.88%). Remarkably, only the class Gammaproteobacteria was identified as the major class (94.44%) among the Proteobacteria population followed by Betaproteobacteria (5.55%) (Supplementary Table 3). Furthermore, the five antimicrobial bacterial isolates RRC48 (Pseudomonas sp.), RRC23 (Pseudomonas frederiksenensis), RRC36 (Flavobacterium sp.), RRC38 (Rhodococcus sp.) and RRC75 (Rhodococcus sp.) were identified under the phylum Proteobacteria, Bacteroidetes and Actinobacteria, respectively (Fig. 4).

Antimicrobial activities of cave bacteria

Five bacterial isolates were observed to exhibit antimicrobial activities. RRC23 (close homologue: Pseudomonas frederiksenensis, 99.77% identity) and RRC75 (close homologue: Rhodococcus sp., 98.85% identity) showed antimicrobial activities against Escherichia coli #15-318 while RRC48 (close homologue: Pseudomonas sp., 84.58% identity) exhibit against Staphylococcus aureus (MRSA). RRC36 (close homologue: Flavobacterium sp., 97.70% identity) and RRC38 (close homologue: Rhodococcus sp., 100% identity) showed antimicrobial activities against the non-pathogenic Staphylococcus aureus strain (Table 2). Intriguingly, RRC48 showed a low identity (84.58%) and query coverage (68%) to its close homologue Pseudomonas sp. and therefore was not deposited to the Genbank or DDBJ.

All these bacteria that exhibited antimicrobial activities were isolated from sample location 2 (RRC23), sample location 3 (RRC36 and RRC38), sample location 4 (RRC48) and sample location 7 (RRC75). Notably, all these sampling sites, besides sample location 2 at the Station 108, were from Station 17 and Station 44, adjacent to the cave entrance (Fig. 1) (Table 1).

DISCUSSION

Raspberry Rising Cave is karstic cave, located in the Glacier National Park’s Tupper mountain system in British Columbia, Canada. Seven locations in the cave were identified from where cave sediments/wall scrapings were sampled. The samples were processed and a cultivation-/PCR-based study were carried out for the first time to elucidate the bacterial diversity of this cave that revealed 103 bacterial isolates. Notably, the samples were incubated at 8°C, although, the temperature of the cave was measured as 4.8°C. A higher incubation temperature was employed as cave bacteria can exhibit confluent growth at temperatures in the range of 5-45°C (Laiz Trobajo et al., 2003).

The cave typically showed the presence of yellow mat/microbial communities (Fig. 2), commonly observed both in karstic and lava caves (Porca et al., 2012; Velikonja et al., 2014; Riquelme et al., 2015). Our study, though preliminary, has identified different bacterial strains with Proteobacteria as the major phylum followed by Actinobacteria and Bacteroidetes. These findings were in consistent to a few previous cultivation-based studies on bacterial phylogenetic diversity of the karstic caves where Proteobacteria was recognized as a predominant bacterial population (Barton et al., 2004; Lu et al.,
Proteobacteria phylum was dominated by the class Gammaproteobacteria with Pseudomonas sp. observed as the major genera in accordance to previous studies (Barton et al., 2004; Banerjee & Joshi, 2016; Yasir, 2018). Proteobacterial population seldom observed to be the dominant class in most of the cave bacterial diversity studies with the majority of the bacterial population has been shown as Actinobacteria (Axenov-Gribanov et al., 2016; Ghosh et al., 2016, 2017; Lavoie et al., 2017). However, a few studies have reported Proteobacteria as the most abundant phylum (Sauro et al., 2018; Yasir, 2018; Barron et al., 2010). Our study has revealed 94.44% of the proteobacterial community was represented by the class Gammaproteobacteria. These findings were consistent with the previous cultivation-based studies where class Gammaproteobacteria were shown to constitute the major bacterial population (Banerjee & Joshi, 2016; Yasir, 2018). For instance, bacterial diversity study on the Meghalayan caves in North-East India, exposed thirty-two different cultivable bacterial species belonging to sixteen different genera where majority belongs to Pseudomonas and Bacillus (Banerjee & Joshi, 2016). Furthermore, we have reported Betaproteobacteria as the less abundant class representing Janthinobacterium sp. (2.91% of the total population) as the only genus (Fig. 4). These observations were in line with previous studies where paired end Illumina was conducted on the bacterial diversity using the V3 region of the 16S DNA from five unknown and unexplored caves of Mizoram, the Northeast India, showing Alpha- and Gammaproteobacteria, the dominant phyotypes while Betaproteobacteria constitute the minor population (De Mandal et al., 2017). Likewise, another study conducted on the microbial diversity and functionality on silica mobility in orthoquartzite caves also reported Janthinobacterium as the less abundant genus among the betaproteobacterial population (Sauro et al., 2018). Our study has reflected a previous literature that has shown Rhodococcus to be the abundant genera among the Actinobacteria (De Mandal et al., 2015), although, in most of the cases Rhodococcus is a rare Actinobacteria (Groth et al., 1999, Adam et al., 2018). Phylum Bacteroidetes with the genus Flavobacterium has been identified as the minor population in our study as stated in the previous findings (Rusznyak et al., 2012; Carmichael et al., 2013).

Five bacterial isolates (RRC23, RRC36, RRC38, RRC48, RRC75) exhibited antagonistic activities against the multidrug resistant strains of E. coli and Staphylococcus aureus revealed in our study. Most of
Fig. 4. Maximum Likelihood tree showing the phylogenetic position of the Raspberry Rising Cave bacterial isolates. The 16S ribosomal operons were obtained from the NCBI database, aligned by MUSCLE with default parameters and the phylogenetic dendrogram was constructed using MEGA6 by neighbor-joining method. Bootstrap confidence levels of 1000 re-samplings were indicated at the nodes. The alphanumeric characters in the parenthesis specifies the gene accession numbers. The isolates marked with a single asterisk indicate lower homology towards their closest relatives (accession numbers were not assigned) while the double asterisks exhibit the isolates with antimicrobial properties. RRC is the abbreviation used for Raspberry Rising Cave.
these isolates belonged to the phylum Actinobacteria and Proteobacteria. RRC38 and RRC75 were both identified as the closest homologues to Actinobacteria (Genus: Rhodococcus sp.) while RRC 23 and RRC 48 as Gammaproteobacteria (Genus: Pseudomonas). Earlier studies have shown that Actinobacteria to be a promising resource for the bioactive compounds (Ghosh et al., 2016, 2017; Rangseekaew & Pathom-Aree, 2019). The genera Streptomyces predominantly exhibited antagonistic effect against bacteria and fungi (Ghosh et al., 2017, Rangseekaew & Pathom-Aree, 2019). For instance, a study on an Italian cave, Grotta dei Cervi, have shown bioactive compounds Cervimycin A, B, C, and D, extracted from Streptomyces tendae strain HKI 0179, exhibited antagonistic effect against Gram-positive bacteria (B. subtilis and S. aureus) as well as multidrug resistant S. aureus (MRSA), vancomycin-resistant Enterococcus faecalis (VRE) and efflux-resistant S. aureus EIS4 (Herold et al., 2005). However, the genera Rhodococcus were not reported expansively from the cave habitat to possess antimicrobial activities. An earlier study has demonstrated isolation of Rhodococcus sp. from the limestone deposit sites in Hundung, Manipur, India, that only showed biocontrol activity against the rice fungal pathogen (Nimaichand et al., 2015). Pseudomonas sp. was rarely identified to exhibit antimicrobial activities from the cave environment. For instance, a previous study has reported Pseudomonas fluorescens, isolated from the Magura Cave, Bulgaria, has exhibited antimicrobial effect against P. aeruginosa and Rhodotorula mucilaginosa displaying 20 mm and 16 mm as the zone of inhibition respectively (Tomova et al., 2013; Ghosh et al., 2016). Our study has identified RRC 36 (close homologue: Flavobacterium sp.) to exhibit antibacterial activities against S. aureus. There is no study accounted until date for the antibacterial activities of Flavobacterium from the cave environment. However, a cultivation-based study from the Antarctic environments has reported antmycobacterial activities of Flavobacterium against Mycobacterium smegmatis and M. tuberculosis (Mojib, et al., 2010). Notably, it has also been observed that all the five isolates exhibited antimicrobial activities were retrieved from all the three stations of the cave (17, 44, and 108) implying that the antimicrobial properties were relatively wide spread in terms of sampling areas and characteristics of the cave sediments within the cave. In addition, the antimicrobial activities were observed at 8°C. However, a previous study has shown Antarctic bacteria produce antimicrobials at low temperature during their growth cycle for their competitive survival (O’Brien et al., 2004).

Taken together, our study has expanded a new understanding to the Canada’s underground. To the best of our knowledge, this is the first attempt that intend to provide the in situ cultured bacterial diversity and antimicrobial activities from the Raspberry Rising Cave. Moreover, the antimicrobial activities exhibited by lesser studied genera such as Rhodococcus, Pseudomonas and Flavobacterium rather than the commonly studied Streptomyces has further opened new frontiers in antimicrobial research studies. Further investigations should emphasize to reveal the whole genome sequences, functional genomics, biochemical assays, fermentation structure elucidation, active component extraction and mode of action of these bacterial isolates in order to understand the underpinning mechanisms of their antimicrobial activities. However, our study gave a smaller snapshot of this cave habitat. Therefore, future study should focus on the metagenomic approaches to have holistic taxonomical and functional profiles of Raspberry Rising Cave microbiomes, in a way to bio-prospect antimicrobial genes/molecules of biotechnological and pharmaceutical relevance and further to elucidate the molecular mechanisms related to microbe-mineral interactions in cave.

ACKNOWLEDGEMENTS

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Raspberry Rising Cave's antibacterial microbes

Nimaichand S., Devi A.M., Tamreihao K., Ningshoujam D.S. & Li W.J., 2015 – Actinobacterial diversity in limestone deposit sites in Hunday, Manipur (India) and their antimicrobial activities. Frontiers in Microbiology, 6: 41. https://doi.org/10.3389/fmicb.2015.00413


INTRODUCTION

Crevice-type caves (CTCs) qualify as a type of pseudokarst caves and belong among frequent landforms of various types of slope failures (Margielewski & Urban, 2017). They originated by a gravitationally induced disintegration of rock massif (Vítek, 1983). Within the research of the CTCs, primarily geological, geomorphological, and geophysical studies are usually conducted (Finlayson, 1986; Self, 1990; Demek & Kopecký, 1996; Pánek et al., 2011; Lenart et al., 2014; Margielewski & Urban, 2017; Tábořík et al., 2017) to provide important data on slope deformation (e.g., material type, structure, depth, velocity of movement). Our understanding of the CTCs can also be significantly complemented by their microclimatic investigation. This kind of research within the CTCs has so far been limited to measurements of temperature and humidity, the results being used especially for speleological exploration (Lenart, 2012) and the monitoring of bats (Wagner et al., 1990). Later, temperature observation has also become a part of landslide geotechnical monitoring instrumentation (Baroň et al., 2003; Klimes et al., 2012).

However, one of the crucial factors controlling cave microclimate is represented also by cave ventilation (Geiger, 1966; Cigna, 1968), a variable that is often being neglected. Cave airflow studies frequently serve for the management and conservation of show caves (Fernandez-Cortes et al., 2006; Russell & MacLean, 2008), optimization of speleotherapy (Faimon & Lang, 2013), or investigation of speleothems. Cave ventilation co-determines the physical and chemical state of the cave atmosphere via changes in microclimatic variables and consequently governs (i)
spearoither growth/destruction and (ii) the chemical
and isotope content of speleothems. The latter factor
is utilized when performing paleoenvironmental
reconstructions based on studies of speleothems. A
thorough understanding of cave ventilation is therefore
required if reliable paleoproxies are to be obtained
from speleothems (Mattey et al., 2010; Baker et al.,
2014). Despite their less frequent occurrence within
the CTCs, speleothems have already been analyzed by
means of the $^{14}$C and U-series dating methods in a
number of CTCs, with the results helping to decipher
landslide ages in some areas (e.g., Pánek et al., 2009;
Farrant et al., 2014; Urban et al., 2015; Lenart et al.,
2018). However, unlike with karst caves, there is a
lack of studies dealing with the ventilation of any type
of pseudokarst caves.

In this paper, a basic qualitative and quantitative
assessment of the cave airflow and ventilation
mechanism of the Velká Ondrášova Cave (VOC) is
provided, aiming to be an introductory study of the
ventilation regime of an exemplary CTC. Airflow
routes within the entire cave were determined by
using an inert chemical tracer. The cave airflow
velocity was being systematically measured during
nine individual monitoring sessions. Furthermore,
wind intensity was also being recorded on the
surface. As a supportive tool for the airflow analysis,
temperature monitoring in both long-term and short-
term modes was carried out. Employing the airflow
oscillations under investigation, the suitability of
the Helmholtz resonator for an explanation of the
ventilation instability has been examined. An insight
into the airflow mechanism of the cave is provided by
performing a set of selected statistical analyses. These
are focused on two predictors: (i) the temperature
difference between inner and outer cave air and (ii)
the outdoor wind. Although there are other possible
triggers of cave ventilation (e.g., pressure changes)
that can be considered as a driving force of cave
ventilation, they are not the subject of the present
study.

**VELKÁ ONDRÁŠOVA CAVE**

The study site is located on the northwestern spur
of Lysá hora (1,323 m a.s.l.), which is the highest
peak of the Moravian-Silesian Beskids, formed by the
Cretaceous gently inclined flysch beds of the Outer
Western Carpathians in Czechia (Fig. 1). The cave
entrance, accessible at 920 m a.s.l., is situated at
the southeastern termination of the distinct double-
crested ridge in the upper part of the vast deep-
seated Lukšíneck landslide dated to 3.5–5 ka BP by
$^{10}$Be (Brézyný et al., 2018). The Velká Ondrášova Cave
(VOC) represents a typical dilation-type (describing
formation mechanism) and initial-type (describing
morphogenesis) crevise-type cave according to the
classification provided by Margielewski & Urban
(2017). Beyond a narrow cross-section (~0.54 m$^2$)
of the entrance part, which was used for microclimate
monitoring sessions and which leads to the distinctly
vast Entrance Dome (ED), the cave splits up into
two morphologically different parts – the Left Branch
(LB) and the Right Branch (RB), both composed of a
step-like system of interconnected abysses and
domes (Lenart et al., 2014). Being the topmost level of
the LB, the Upper Shaft is situated shallow below
the surface and sporadically changes into a boulder
cave *sensu* Margielewski & Urban (2017). The bottom
of the mapped system is situated in the RB, 35 m
below the entrance. The known cave corridors reach
a cumulative length of 217 m (Wagner et al., 1990).

The origin of the mass movement controls the
dynamic temperature regime of the cave. Although
the cave is accessible only through one entrance,
we assume there exist many other narrow openings
represented by gravitationally widened joints or inter-
boulder gaps.

The external annual air temperature of the area is
~3°C and the average 211 days with rainfall result
in total annual precipitation exceeding 1,400 mm
(climatic data from the Lysá hora Weather Station,
provided by Czech Hydrometeorological Institute,
2019). As a protected bat wintering site, the cave is
closed by a lockable bar and is visited only by cavers
performing bat monitoring.

**METHODS**

The study of the cave airflow within the Velká
Ondrášova Cave was performed using the following
three approaches: (i) a qualitative assessment of
airflow within the cave by means of a chemical
tracer, (ii) auxiliary air temperature monitoring
inside and outside the cave environment, and (iii)
airflow velocity measurement inside and outside the
cave. The microclimatic data were obtained during
long-term continuous measurement and monitoring field
sessions. The field sessions provided both temperature and airflow short-term data for a statistical and spectral analysis dealing with the cave airflow mechanism. During the long-term measurement, only temperature data were collected, illustrating the temporal changes of a variable closely connected with the cave airflow. The processing and analysis of time series data were performed using the STATISTICA 10 software (TIBCO Software Inc., 2019).

Temperature monitoring

Air temperature within the cave was continually measured from December 2012 to February 2013 and from July 2013 to November 2014 with CS02 dataloggers (Petr Holub, measuring range from −50°C to +50°C, resolution 0.06°C, accuracy ±0.5°C) with a 1-hour time step. Some of these data were compared with the mean daily air temperature data from the Lysá hora Weather Station (Czech Hydrometeorological Institute, 2019). The temperature sensors were placed in three different parts of the cave (see Fig. 2 for locations of the loggers): (i) the ED, a shallow part of the cave near the entrance, (ii) the bottom of the LB, (iii) the bottom of the RB, the deepest accessible point of the Velká Ondrášova Cave, ~35 m below the entrance level.

The on-the-spot temperature gauging within the monitoring sessions was performed with a WS8610 thermometer (Garni technology, measuring range from −30°C to +70°C, resolution 0.1°C, accuracy ±1°C), logging the data with a 5-min time step into the built-in datalogger. During the sessions, the thermometer sensors were placed at the following positions (the corresponding variables are indicated in the parentheses): (i) outside the cave, −15 m from the cave entrance to avoid the thermal influence of the cave on the measurement ($T_{out}$); (ii) outside the cave, in close proximity of the cave entrance ($T_{out}^*$); (iii) in the Entrance Dome, matching the sensor location of the continual temperature monitoring ($T_{in}$).

The temperature of the cave air ($T_f$) flowing across the narrowed cross-section situated behind the entrance was recorded with a thermistor included in the AM-4214SD thermo-anemometer (Lutron, measuring range from −50°C to +1300°C, resolution 0.1°C, accuracy ±0.4% + 0.5°C). Based on the monitoring, the temperature gradient $\Delta T$ was determined as a difference between the temperatures measured inside and outside the cave (i.e., $\Delta T = T_{in} - T_{out}$). Similarly, the temperature gradient $\Delta T^*$ (i.e., $\Delta T^* = T_{in} - T_{out}^*$) was defined, influenced by the closeness of the cave entrance.

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Fig. 2. Ventilation pattern of the Velká Ondrášova Cave, recorded in winter 2013; marked on the cave plan by Wagner et al. (1990); the cross-sections out of scale.


Ventilation monitoring

The ventilation pattern of the whole length of the Velká Ondrášova Cave was investigated by determining the approximate direction and intensity of airflow movement using an airflow tester kit (Dräger Safety). It includes an aspirator bulb, which blows the air into the testing tube filled with sulfuric acid, exuding a temperature-neutral chemical tracer that makes the air movement visible on the testing site (Fig. 3). The spatial distribution of the air mass movement throughout the cave was observed by means of this technique in summer 2012 and winter 2013.

Fig. 3. Detection of directions and approximate intensity of the air movement in the Right Branch of the Velká Ondrášova Cave (photo by J. Lenart).

There were six monitoring sessions between February 2015 and April 2018, one in August 2015, and two more in March 2018. They involved a measurement of the cave airflow velocity (AF<sub>in</sub>) within the narrow cross-section beyond the entrance (see Fig. 2 for the position) and of the wind velocity (AF<sub>out</sub>) recorded outside the cave. The cave airflow was measured with the AM-4214SD thermo-anemometer sensor (measuring range from 0.06 to 20 m·s<sup>−1</sup>, resolution 0.01 m·s<sup>−1</sup>, accuracy ±5%), sampled with a 5-sec time step with a built-in datalogger. In order to get an idea about the ventilation, the linear velocity of the cave airflow (m·s<sup>−1</sup> units) was consecutively recalculated into volume velocity (m<sup>3</sup>·s<sup>−1</sup> units), counting the flow area of ~0.54 m<sup>2</sup>, otherwise, linear velocity was utilized for analysis.

The wind velocity outside the cave was gauged with a M309 mechanic anemometer (TFA Dostmann, measuring range from 0.2 to 30 m·s<sup>−1</sup>, resolution 0.1 m·s<sup>−1</sup>, accuracy ±5%), mounted on a photographic tripod ~1.3 m above the ground, and recorded with a camera for later reading off with a 5-sec time step. Time synchronization of all the monitoring devices during the sessions was ensured with a DCF-77 radio signal reception.

RESULTS AND ANALYSIS

Ventilation pattern

A qualitative assessment of the ventilation within the cave took place twice, in summer and winter: (i) on June 1, 2012, in conditions of ΔT ~−15.1°C and (ii) on February 28, 2013, when ΔT ~3.4°C. During the first observation in summer, no perceptible air currents were detected in the cave interior, except for the airflow identified within superficial parts of the cave and in the entrance. The results of the second investigation in winter are presented in Figure 2. The ventilation of the Upper Shaft and the shallow levels of the cave tends to be rather weak to perceptible and horizontally oriented, while the deeper levels of the cave and the bottommost parts of the branches are characterized by mainly very weak horizontal currents and vertical upward currents. Horizontal currents are almost absent or very weak in the deep levels of the LB, where downward currents were also detected. Although the ED and the wider crevices in the LB and in the Upper Shaft seem to be static, weak air currents flow along their walls.

Long-term temperature data

Along-standing monitoring of the cave air temperature was carried out during winter 2012/2013 (henceforth the winter monitoring) and between July 2013 and November 2014 (henceforth the annual monitoring). The resulting data from the winter monitoring within three cave sites (parts of the ED, LB, and RB) are compared with the outside temperature in Figure 4 (for locations of the loggers see Fig. 2). While the outside temperature fluctuated between −12.9°C and 8.0°C within the winter data, the RB proved to be the most stable part of the cave with a mean temperature of 3.2°C ±0.3°C. The LB appeared to be slightly more dynamic with a temperature ranging from 1.1°C to 4.8°C. Based on the winter data, the ED seems to be quite steady, despite its relative proximity to the cave entrance.

During the annual monitoring, only the data from the ED and the RB are available due to loss of the logger located in the LB. A comparison of the ED and LB air temperatures recorded during the annual monitoring is shown in Table 1 and Figure 5. During the annual period, the ED is characterized by a temperature range from 2.4°C to 12.1°C with a mean value of 6.3°C ± 2.6°C. Compared with the RB, the ED
data reflect a strong seasonality. An annual amplitude of almost 10°C contrasts with the stable and for most of the year colder microclimate of the bottommost part of the RB, characterized by an annual amplitude of 1°C and an average temperature of 3.2°C ±0.2°C.

### Table 1. Descriptive statistics of the annual monitoring data (temperature) from two monitoring sites within the Velká Ondrášova Cave – Entrance Dome and Right Branch.

<table>
<thead>
<tr>
<th></th>
<th>Entrance Dome</th>
<th>Right Branch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean [°C]</td>
<td>6.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Median [°C]</td>
<td>6.4</td>
<td>3.2</td>
</tr>
<tr>
<td>Mode [°C]</td>
<td>2.7</td>
<td>3.3</td>
</tr>
<tr>
<td>Standard deviation [°C]</td>
<td>2.6</td>
<td>0.2</td>
</tr>
<tr>
<td>Variance</td>
<td>6.7</td>
<td>0</td>
</tr>
<tr>
<td>Coefficient of variation</td>
<td>0.41</td>
<td>0.07</td>
</tr>
<tr>
<td>Range [°C]</td>
<td>9.7</td>
<td>1</td>
</tr>
<tr>
<td>Minimum [°C]</td>
<td>2.4</td>
<td>2.7</td>
</tr>
<tr>
<td>Maximum [°C]</td>
<td>12.1</td>
<td>3.7</td>
</tr>
<tr>
<td>Kurtosis</td>
<td>−1.3</td>
<td>−0.8</td>
</tr>
<tr>
<td>Skewness</td>
<td>0.1</td>
<td>0.1</td>
</tr>
</tbody>
</table>

Yielding over 22 hours’ worth of data, nine monitoring field sessions were conducted in various outdoor conditions during 2015 (February 1, 2015; February 7, 2015; February 22, 2015; March 7, 2015; March 28, 2015; April 10, 2015; August 27, 2015; and March 5, 2018) and February 22, 2015; March 7, 2015; and March 2, 2018 were characterized by only random weak wind flurries interrupting quite calm wind conditions.

The average cave airflow velocity $AF_{in}$ fluctuates from 0.27 to 0.61 m·s$^{-1}$ with a maximum reaching up to 1.25 m·s$^{-1}$. In the cross-sectional area of ~0.54 m$^2$, the mean values of volumetric airflow velocity range between 0.15 and 0.35 m$^3$·s$^{-1}$ with a maximum of up to 0.71 m$^3$·s$^{-1}$.

### Field session data

Yielding over 22 hours’ worth of data, nine monitoring field sessions were conducted in various outdoor conditions during 2015 (February 1, 2015; February 7, 2015; February 22, 2015; March 7, 2015; March 28, 2015; April 10, 2015; August 27, 2015; and March 5, 2018) and spring 2018 (March 2, 2018; March 5, 2018; Fig. 6). The mean values and standard deviation of important microclimatic variables measured during the sessions are available in Table 2. Within these sessions, the outdoor temperatures $T_{out}$ ranged from −8.6°C to 22.8°C, implying a fluctuation of the temperature gradient $\Delta T$ between −13.8°C and 11.4°C. The temperature $T_{cave}$ varied from 3.9°C to 5.4°C, with the exception of the summer session on August 27, 2015, when the value of 15.3°C was recorded. During sessions characterized by strong wind conditions outside the cave, increased variability of the temperature of the flowing cave air $T_{cave}$ documented by a heightened standard deviation reaching up to ~0.6°C, correlates with the variance of outdoor wind speed $AF_{out}$ and the cave airflow velocity $AF_{in}$.

$AF_{out}$ was undetectable during two sessions (February 1, 2015 and February 7, 2015), while during the March 28, 2015 session, the highest mean value exceeding 2.85 m·s$^{-1}$ was recorded. Wind gusts often reached up to 4–5 m·s$^{-1}$ with a maximum of 7.3 m·s$^{-1}$ (March 5, 2018). Strong wind conditions with distinct gusts were recorded during the sessions on March 28, 2015; April 10, 2015; August 27, 2015; and March 5, 2018; while the sessions on February 22, 2015; March 7, 2015; and March 2, 2018 were characterized by only random weak wind flurries interrupting quite calm wind conditions.

Cave airflow oscillations

Typical oscillations occur when recording the cave airflow velocity. A detailed mechanism of their origin remains unclear, however, Cigna (1968) and Plummer (1969) suggest that the concept of the Helmholtz resonator could explain the signal oscillations. In theory, the resonator is described as an air reservoir with rigid walls and defined geometry. The reservoir is vented through a neck with a determined sectional area and reservoir volume. Based on Rothman (1989) and French (2005), the resonance frequency $f$ [Hz] is given by

$$f = \frac{c_s}{2\pi} \sqrt{\frac{A}{L_r V_r}} \quad (1)$$

where $c_s$ is the speed of sound in air (~330 m·s$^{-1}$), $A$, is the cross-section area of the resonator neck [m$^2$], $L_r$ is the length of the resonator neck [m], and $V_r$ is the total volume of the resonator [m$^3$].

Six 15-min segments of cave airflow velocity were selected from the winter/spring 2015 monitoring sessions (February 1, 2015; February 7, 2015; February 22, 2015; March 7, 2015; March 28, 2015; and April 10, 2015) to verify a potential consistency of the taped cave airflow oscillations with the model of the Helmholtz resonator. These signal segments were subjected to Fast Fourier Transform (Rao et al., 2010; Heilbronner & Barrett, 2014) to convert the data and unfold them in frequency domain. Based on the resulting spectral densities and the application of Fisher’s test of periodicity (Fisher, 1929; Siegel, 1980), the procedures have identified over 50 significant periods/frequencies corresponding in particular to intervals of 20–50 s / 50–20 mHz. The spectral density maximum of each of the selected records corresponds to the periods of 24, 32, 39, 180, and 450 sec. The results are shown in Figure 7. Considering the resonator parameters, the highest identified statistically significant frequency $f$ equals ~62.5 mHz (16-sec period), $A_r$ ~0.54 m$^2$, and $L_r$ ~5 m; the calculated cave volume $V_r$ corresponds to ~76,000 m$^3$. When modifying the frequency to 30 mHz (33-sec period), the figured $V_r$ equals ~330,000 m$^3$. 

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Table 2. Mean and standard deviation of the microclimatic variables measured during the monitoring sessions.

<table>
<thead>
<tr>
<th>Monitoring session</th>
<th>$A_{F_{in}}$ [m s$^{-1}$]</th>
<th>$A_{F_{out}}$ [m s$^{-1}$]</th>
<th>$T_{f}$ [°C]</th>
<th>$T_{out}$ [°C]</th>
<th>$\Delta T$ [°C]</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Feb-2015</td>
<td>0.46 ± 0.03</td>
<td>—</td>
<td>5.1 ± 0.0</td>
<td>−1.3 ± 0.4</td>
<td>4.6 ± 0.4</td>
</tr>
<tr>
<td>7-Feb-2015</td>
<td>0.44 ± 0.03</td>
<td>—</td>
<td>4.9 ± 0.0</td>
<td>−7.8 ± 0.1</td>
<td>11.1 ± 0.1</td>
</tr>
<tr>
<td>22-Feb-2015</td>
<td>0.27 ± 0.07</td>
<td>0.14 ± 0.34</td>
<td>5.4 ± 0.2</td>
<td>3.9 ± 0.7</td>
<td>−0.6 ± 0.7</td>
</tr>
<tr>
<td>7-Mar-2015</td>
<td>0.37 ± 0.04</td>
<td>0.09 ± 0.24</td>
<td>5.0 ± 0.1</td>
<td>0.6 ± 0.4</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>28-Mar-2015</td>
<td>0.31 ± 0.12</td>
<td>2.85 ± 0.83</td>
<td>3.9 ± 0.6</td>
<td>0.6 ± 0.4</td>
<td>2.7 ± 0.4</td>
</tr>
<tr>
<td>10-Apr-2015</td>
<td>0.36 ± 0.14</td>
<td>2.24 ± 0.83</td>
<td>4.6 ± 0.2</td>
<td>9.2 ± 0.5</td>
<td>−6.0 ± 0.5</td>
</tr>
<tr>
<td>27-Aug-2015</td>
<td>0.40 ± 0.21</td>
<td>1.40 ± 0.60</td>
<td>15.3 ± 0.5</td>
<td>22.8 ± 0.4</td>
<td>−13.8 ± 0.4</td>
</tr>
<tr>
<td>2-Mar-2018</td>
<td>0.61 ± 0.03</td>
<td>0.32 ± 0.45</td>
<td>4.2 ± 0.3</td>
<td>−8.6 ± 0.1</td>
<td>11.4 ± 0.1</td>
</tr>
<tr>
<td>5-Mar-2018</td>
<td>0.56 ± 0.18</td>
<td>2.59 ± 1.12</td>
<td>4.1 ± 0.1</td>
<td>−0.4 ± 1.5</td>
<td>2.9 ± 1.5</td>
</tr>
</tbody>
</table>

$A_{F_{in}}$ – cave airflow velocity, $A_{F_{out}}$ – speed of outdoor wind, $T_{f}$ – flowing cave air temperature, $T_{out}$ – outdoor temperature unaffected by proximity of the cave entrance, $\Delta T$ – temperature gradient unaffected by the entrance proximity.

### Regression analysis

Many authors have already shown that cave airflow can be described as a function of density differences between the cave and the outdoor air mass (Cigna, 1968; Cigna & Forti, 1986; Wigley & Brown, 1971; de Freitas et al., 1982; Spötl et al., 2005; Kowalczyk & Froelich, 2010). A rearrangement of the empirical Darcy–Weisbach equation for turbulent flow in pipes enables a definition of the speed of cave airflow as a function of temperature conditions, cave morphology, and its geometry (Atkinson et al., 1983; Lismonde, 2002). Based on simplified assumptions, confirmed by, e.g., Atkinson et al. (1983), Fernández-Cortes et al. (2006), Baldini et al. (2008), or Faimon et al. (2012), cave airflow can also be expressed as a function of the temperature gradient, introducing this variable as an alternative and simplifying airflow predictor.

Our data on airflow were approximated with three relevant regression models, although many more models could be examined, combining more airflow predictors, as follows from de Freitas et al. (1982) or Faimon et al. (2012).

Ohata et al. (1994) and Luetscher & Jeannin (2004) have demonstrated that the speed of cave airflow is proportional to the square root of the temperature gradient $\Delta T$. Therefore, the first model examining this relation is the square root model (SRM),

$$AF_{in} = b_0 + b_1 \sqrt{\Delta T}$$

where $AF_{in}$ represents the speed of cave airflow [m s$^{-1}$] as a dependent variable, $\Delta T$ is the temperature gradient [°C] introduced as an independent variable, $b_0$ is an intercept, and $b_1$ is a coefficient. The second approach is represented by the linear model (LM),

$$AF_{in} = b_0 + b_1 \Delta T$$

$$AF_{in} = b_0 + b_1 \Delta T + b_2 \Delta T^2$$
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Fig. 7. Spectral analysis of selected 15-min segments of the cave airflow data from six monitoring sessions: a) 1-Feb-2015; b) 7-Feb-2015; c) 22-Feb-2015; d) 7-Mar-2015; e) 28-Mar-2015, f) 10-Apr-2015. Hamming weights: 0.0357, 0.2411, 0.4464, 0.2411, 0.0357.

\[
AF_{in} = b_0 + b_1\Delta T 
\]

and, finally, the quadratic model (QM) approximated the airflow data,

\[
AF_{in} = b_0 + b_1\Delta T + b_2(\Delta T)^2
\]

where the additional coefficient \(b_2\) is used. The fitting of the experimental airflow data and the \(b_0, b_1,\) and \(b_2\) calculations were done using the least square method (Gelman & Hill, 2007) (Table 3). However, only the cave airflow data attributed to \(AF_{out} \sim 0\) (zero-valued speed of outdoor wind) enter the analysis (number of observations, \(n = 4,206\)) to avoid any variance of the data caused by a dynamic driver, which is analyzed separately in the next chapter.

Table 3. Parameter estimates of the discussed regression models.

<table>
<thead>
<tr>
<th>Parameter estimates</th>
<th>estimate</th>
<th>SE</th>
<th>t-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>LM</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(b_0)</td>
<td>0.3016</td>
<td>0.0014</td>
<td>215.7</td>
<td>0</td>
</tr>
<tr>
<td>(b_1)</td>
<td>0.0243</td>
<td>0.00033</td>
<td>73.9</td>
<td>0</td>
</tr>
<tr>
<td>(b_0)</td>
<td>0.2135</td>
<td>0.00219</td>
<td>97.4</td>
<td>0</td>
</tr>
<tr>
<td>(b_1)</td>
<td>0.0972</td>
<td>0.00118</td>
<td>82.2</td>
<td>0</td>
</tr>
<tr>
<td>(b_0)</td>
<td>0.2737</td>
<td>0.00107</td>
<td>254.9</td>
<td>0</td>
</tr>
<tr>
<td>(b_1)</td>
<td>0.0517</td>
<td>0.00047</td>
<td>108.8</td>
<td>0</td>
</tr>
<tr>
<td>(b_2)</td>
<td>-0.0032</td>
<td>0.00005</td>
<td>-66.0</td>
<td>0</td>
</tr>
</tbody>
</table>

For a better idea of the problem, the relation of complete cave airflow data to temperature gradient \((n = 15,324)\) is given in Figure 8A; while the regression analysis of filtered data, the model parameters, and the results of analysis of variance (ANOVA) are shown in Figure 8B and in Table 4. Verifying statistical significance, none of the \(p\)-values of the models and their parameters exceed the 0.05 level of significance. Based on coefficients of determination, the best-fitting regression model was the QM \((R^2 = 0.79)\), while less well-fitting values were demonstrated by the SRM \((R^2 = 0.62)\) and the LM \((R^2 = 0.57)\). The intercept value \(b_0\) ranges between \(21.35 \times 10^{-2}\) and \(30.16 \times 10^{-2}\), the coefficient \(b_1\) varies from \(2.43 \times 10^{-2}\) to \(9.72 \times 10^{-2}\), and the single parameter \(b_2\) reaches \(-0.32 \times 10^{-2}\).

Statistical testing

During strong wind intervals, visible water steam was clearly recognized in front of the cave. A brief look on the raw session data suggests the influence of external wind on the cave ventilation. The sessions that logged strong external wind conditions are characterized by a cave ventilation frequently reaching up to \(1 \text{ m} \cdot \text{s}^{-1}\), and by a distinct variance (Fig. 6). The possible connection between the outdoor wind and the cave ventilation is verified in a statistical manner. Therefore, correlation analysis and testing for variance were chosen to examine potential links between the variables recorded during the monitoring sessions.

The dataset containing all recorded and derived variables went through filtering. However, unlike in the regression analysis, only the data attributed to \(AF_{out} > 0\) (n = 5,224) enter the correlation analysis, examining possible relations between the variables \(AF_{out}^*, AF_{out}, T_f, T_{in}, T_{out}, T_{out}^*, \Delta T,\) and \(\Delta T^*\). Representing outliers, data from the summer session on August 27, 2015 were also excluded and analyzed separately.

Examining the \(AF_{out} - AF_{in}\) relation within the filtered data has shown no link between these variables (correlation coefficient \(r = 0.09)\). However, a moderate negative correlation between \(AF_{out}^*\) and the temperature of flowing cave air \(T_f (r = -0.56)\) has emerged within the correlation matrix (Table 5). As has been shown by the analysis, the external wind speed \(AF_{out}^*\) is connected to the outdoor temperature \(T_{out}^* (r = -0.42)\) and the derived temperature gradient \(\Delta T^* (r = 0.42)\). The \(AF_{in} - \Delta T (r = 0.34)\) and \(AF_{in} - \Delta T^*\)
Table 4. Results of the analysis of variance (ANOVA) of the discussed models.

<table>
<thead>
<tr>
<th>Model</th>
<th>R²</th>
<th>Regression</th>
<th>Residual</th>
<th>F-value</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>SS</td>
<td>DF</td>
<td>MS</td>
<td>SS</td>
<td>DF</td>
</tr>
<tr>
<td>LM</td>
<td>0.57</td>
<td>19.76</td>
<td>1</td>
<td>19.7622</td>
<td>15.21</td>
</tr>
<tr>
<td>SRM</td>
<td>0.62</td>
<td>21.56</td>
<td>1</td>
<td>21.5577</td>
<td>13.42</td>
</tr>
<tr>
<td>QM</td>
<td>0.79</td>
<td>27.51</td>
<td>2</td>
<td>13.7537</td>
<td>7.47</td>
</tr>
</tbody>
</table>

Fig. 8. Relationship between cave airflow velocity $A_{F_{in}}$ and temperature gradient $\Delta T$ from field monitoring data: A – all unfiltered session data ($n = 15,324$); B – regression models of filtered data ($n = 4,206$) characterized by zero-valued wind velocity $A_{F_{out}}$. Red-colored data are the selected data fitted with the SRM, QM and LM.

$r = -0.19$ relations were evaluated as weakly correlated. Within the excluded dataset containing the summer data of August 27, 2015, analysis outcomes have pointed out a weak relation of $A_{F_{in}} - A_{F_{out}}$ ($r = 0.36$).

Testing for variance has been used to determine whether the variability of $A_{F_{in}}$ under strong external wind conditions is significantly higher than its variance under calm wind conditions. The signal of cave airflow from session data was separated into two equally sized datasets based on $A_{F_{out}}$. The first dataset represents a signal with non-zero wind velocity ($A_{F_{out}} > 0$), while the other contains data characterized by a zero-valued speed of wind ($A_{F_{out}} \sim 0$). Both the datasets have been tested with an $F$-test for equality of variance, whose results are shown in Table 6. The assessed variance equals 0.017 for the $A_{F_{out}} > 0$ dataset and 0.008 for the $A_{F_{out}} \sim 0$ set. Based on 4,205 observations, the $F$-test supports the alternative hypothesis that the variances of both datasets are not equal at 0.05 significance level. It is worth noting that the average of $A_{F_{in}}$ within the $A_{F_{out}} > 0$ set is 0.32 m·s$^{-1}$, while within the $A_{F_{out}} \sim 0$ set, the average $A_{F_{in}}$ reaches 0.38 m·s$^{-1}$.

Table 5. Correlation matrix of the logged and derived variables from the filtered session data.

<table>
<thead>
<tr>
<th>$A_{F_{in}}$</th>
<th>$T_{f}$</th>
<th>$T_{in}$</th>
<th>$T^{*}_{out}$</th>
<th>$T_{out}$</th>
<th>$\Delta T^{*}$</th>
<th>$\Delta T$</th>
<th>$A_{F_{out}}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$A_{F_{in}}$</td>
<td>1.00</td>
<td>-0.22</td>
<td>-0.60</td>
<td>0.19</td>
<td>-0.36</td>
<td>-0.19</td>
<td>0.34</td>
</tr>
<tr>
<td>$T_{f}$</td>
<td>-0.22</td>
<td>1.00</td>
<td>0.29</td>
<td>0.73</td>
<td>0.52</td>
<td>-0.73</td>
<td>-0.51</td>
</tr>
<tr>
<td>$T_{in}$</td>
<td>-0.60</td>
<td>0.29</td>
<td>1.00</td>
<td>0.00</td>
<td>0.63</td>
<td>0.00</td>
<td>-0.60</td>
</tr>
<tr>
<td>$T^{*}_{out}$</td>
<td>0.19</td>
<td>0.73</td>
<td>0.00</td>
<td>1.00</td>
<td>0.88</td>
<td>-1.00</td>
<td>-0.88</td>
</tr>
<tr>
<td>$T_{out}$</td>
<td>-0.36</td>
<td>0.52</td>
<td>0.63</td>
<td>0.88</td>
<td>1.00</td>
<td>-0.88</td>
<td>-1.00</td>
</tr>
<tr>
<td>$\Delta T^{*}$</td>
<td>-0.19</td>
<td>-0.73</td>
<td>0.00</td>
<td>-1.00</td>
<td>-0.88</td>
<td>1.00</td>
<td>0.88</td>
</tr>
<tr>
<td>$\Delta T$</td>
<td>0.34</td>
<td>-0.51</td>
<td>-0.60</td>
<td>-0.88</td>
<td>-1.00</td>
<td>0.88</td>
<td>1.00</td>
</tr>
<tr>
<td>$A_{F_{out}}$</td>
<td>0.09</td>
<td>-0.56</td>
<td>-0.01</td>
<td>-0.42</td>
<td>0.01</td>
<td>0.42</td>
<td>-0.02</td>
</tr>
</tbody>
</table>

$A_{F_{in}}$ – cave airflow velocity, $T_{f}$ – flowing cave air temperature, $T_{in}$ – cave air temperature (Entrance Dome), $T^{*}_{out}$ – outdoor temperature measured in front of the cave, $T^{*}_{out}$ – outdoor temperature unaffected by proximity of the cave entrance, $\Delta T^{*}$ – temperature gradient influenced by the entrance proximity, $\Delta T$ – temperature gradient unaffected by the entrance proximity, $A_{F_{out}}$ – speed of outdoor wind.

Table 6. Results of the $F$-test of equality of variance: $F$ – test statistic, $F_{crit}$ – critical test statistic, $P (F \leq f)$ – probability of null hypothesis truthfulness.

<table>
<thead>
<tr>
<th>$A_{F_{out}} &gt; 0$</th>
<th>$A_{F_{out}} \sim 0$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean [m·s$^{-1}$]</td>
<td>0.32</td>
</tr>
<tr>
<td>Variance</td>
<td>0.017</td>
</tr>
<tr>
<td>Frequency</td>
<td>4205</td>
</tr>
<tr>
<td>$F$</td>
<td>2.06</td>
</tr>
<tr>
<td>$P (F \leq f)$</td>
<td>0.017</td>
</tr>
<tr>
<td>$F_{crit}$</td>
<td>1.05</td>
</tr>
</tbody>
</table>
DISCUSSION

Airflow pattern and magnitude

In this study, we used a chemical tracer to visualize a low-velocity air movement and to map the airflow routes within the VOC. Besides utilizing diverse tracers (Halbert & Michie, 1971), other different methods can be used for airflow detection, such as the application of laser light sheet technique (Magne et al., 2017) or the use of neutral buoyancy balloons (de Freitas et al., 1982).

The results have shown a predominantly static character of most of the cave. In winter, the relatively warmer air mass within the cave tends to be transferred by numerous vents out of the cave. Although undetectable within most of the cave length in summer, the airflow was distinct within the shallowest parts of the cave. The monitoring session on August 27, 2015, executed in conditions of ΔT ~ −13.8°C, recorded a magnitude and variability of airflow comparable with data from winter sessions. Being affected by external wind, the ventilation regime of the VOC cannot be assessed by the traditional microclimatic classification by Geiger (1966). It follows from the complex cave anatomy that there are a number of vents above the cave, enabling an intensive bidirectional energy-mass exchange between the cave and its outer environment. It results in a rather dynamic temperature regime of the cave, as confirmed by over one-year-long continual temperature monitoring in the ED, pointing to an annual temperature amplitude of ~10°C. In contrast, the deep parts of both the LB and the RB manifest a stable microclimate, as documented by a stagnant airflow and a total temperature amplitude below 1°C. Comparing the LB and the RB, the former turns out to be a little more dynamic than the latter, a fact confirmed by the previous monitoring performed by Lenart (2012) in 2009 and 2010. The RB is isolated within the central part of the ridge and reaches deeper levels of the rock massif (the temperature monitoring site is located ~35 m deep), while the relatively shallower passages of the LB run out southward quite close to the gravitationally induced rocky trench, situated ~20 m lower within the southwestern slope.

The mean cave airflow velocity \( \Delta F_{cave} \) reached values between 0.27 and 0.61 m\( \cdot \)s\(^{-1} \), with a maximum exceeding 1.25 m\( \cdot \)s\(^{-1} \), being comparable with similar values recorded in other caves, e.g., Hollow Ridge Cave in Florida, USA (Kowalczyk & Froelich, 2010); Fuji Ice Cave in Japan (Ohata et al., 1994); or King Solomons Cave in Tasmania (Russel & MacLean, 2008). The absolute values of the velocity exceed the rates of numerous known caves, e.g., Niedźwiedzia Cave in Poland (Pflitsch & Piasecki, 2003); Kartchner Caverns in Arizona, USA (Buecher, 1999); or the Císařská Cave in the Czech Republic (Faimon et al., 2012). However, e.g., Pflitsch et al. (2010) reported airflow rates reaching over 6 m\( \cdot \)s\(^{-1} \) in S & G Cave in South Dakota (USA) and an airflow velocity rising up to almost 10 m\( \cdot \)s\(^{-1} \) was recorded within the Vjetrenica (Windy) Cave in Herzegovina (Milanović, 2018). Bögli (1980) reported airflow velocity over 46 m\( \cdot \)s\(^{-1} \) measured in the Pnargözü Cave (Turkey), exceeding the airflow velocity maximum of the VOC almost thirty-seven times.

As implied from the analysis, ventilation rates of the VOC during active ventilation regime correspond to 540–1,260 m\(^3\)\( \cdot \)h\(^{-1} \), amounting to ~13,000–30,000 m\(^3\) per day. This result is comparable with data from Kartchner Caverns in Arizona, USA (Buecher, 1999), where the values of ~12,000 m\(^3\) per day were recorded. In contrast, air mass with the rates of 7,260 m\(^3\)\( \cdot \)h\(^{-1} \), corresponding to ~175,000 m\(^3\) per day, is supposed to be ventilated in the Buddhist Cave, China (Christoforou et al., 1996). The values of 48,600 m\(^3\)\( \cdot \)h\(^{-1} \) corresponding to ~1,160,000 m\(^3\) per day, were reported by Freitas et al. (1982) from the Glowworm Cave, New Zealand. However, owing to the heavily disintegrated rock environment of the VOC (Lenart et al., 2014), it may vent much more air mass by other conduits, represented by numerous cracks and relaxed zones within the rock massif above the cave. Thus, the above determined ventilation is related to just one vent and needs to be taken for a minimum value.

Cave airflow oscillations

Resonance of cave airflow has already been analyzed by many authors in the past (e.g., Moore & Nicholas, 1964; Eckler, 1965; Peters, 1965; Cigna, 1968; Plummer, 1969; Russel, 1974), recently in more detail by Bérest et al. (1999), Badino (2010), Faimon et al. (2012), Lang & Faimon (2012), and this phenomenon has been examined even on Mars (Williams et al., 2017). A prediction of cave volume and structure by means of the Helmholtz resonator seems to be feasible in certain cases (Plummer, 1969; Rothman, 1989). Spectral analysis applied to the cave airflow signal has detected multiple different frequencies, questioning the appropriateness of the resonance model applied to the VOC, since the cave resonance would produce only one principal frequency. According to assumptions based on the cave mapping by Wagner et al. (1990, Fig. 2), the volume inferred from the resonance model (~76,000 m\(^3\)) by applying the highest traced frequency seems to be meaningless. Modifying the model parameters in reasonable ranges does not cause the figured volume to approach its real value.

There are several aspects that could cause the model to fail: (i) the complex morphology of the cave, unsuitable for a definition of the reservoir geometry (a disputable cross-section and length of the reservoir neck), (ii) heavily fractured rock massif with numerous cracks blocked by different-sized colluvial material, resulting in multiple vents in the reservoir, (iii) evident dependence of the cave ventilation on external wind, suggesting that the variation of the external wind intensity could be responsible for the oscillations. Faimon et al. (2012) and Lang & Faimon (2012) mention similar factors disabling the model applicability in the case of the Císařská Cave (Czechia) and consider the fluctuating temperature gradients near the cave entrances as the primary cause of the
oscillations. In the case of the VOC, accumulation and wind-induced dispersion of the warm air mass in front of the cave entrance, responsible for the temperature variability, has been also observed. Their presence is documented by results of the correlation analysis, i.e., negatively correlated $T_{f} - \Delta T^{*}$ and positively correlated $AF_{\text{out}} - \Delta T^{*}$ links (see below).

**Airflow as a function of the temperature gradient**

Various models of cave airflow have already been proposed by a number of authors, including changes in inner/outer air temperature and/or air densities, cave wall temperature, atmospheric pressure, frictional properties of the cave for fluid flow, or site-specific geometric factors of the cave (e.g., de Freitas et al., 1982; Christoforou et al., 1996; Kowalczyk & Froelich, 2010; Faimon et al., 2012). In this study, using the same simple independent variable, three different regression models were compared with each other, confirming the dependence of the cave airflow on the temperature gradient $\Delta T$. Indicating statistically significant relations at the 95% confidence level for all models, the LM accounts for 57% of total variance of the airflow data, while the SRM elucidates 5% more, documenting the usability of the simplified Darcy–Weissbach equation. Faimon & Lang (2013) reported a similar fitting of the SRM, explaining the ventilation within the Čísařská Cave. Ohata et al. (1994) and Luetscher & Jeannin (2004) proved the suitability of the SRM with better results, applying the model to caves with different geometries.

Nonlinearity of the airflow data was highlighted by the best-fitted QM, defining almost 80% of the airflow data variance. The QM suggests the cave airflow to culminate at $\sim 0.48 \, \text{m} \cdot \text{s}^{-1}$, when $\Delta T$ reaches $\sim 8^\circ \text{C}$. According to this model, further $\Delta T$ increase could cause the cave ventilation to be attenuated. The natural nonlinearity of the Darcy–Weissbach equation explains this effect only partially (Jeannin, 2001). The decrease in ventilation may be explained by possible unequal cooling of the shallowerest cave parts. The strongly fractured and disintegrated rock massif makes the air mass exchange between the outside and the shallow cave parts very intense, resulting in unequal cooling of some superficial segments and the successive slowing down of the ventilation. If time-delayed or unrepresentative cave air temperature $T_{\text{m}}$ (not reflecting the cooling of the ventilated superficial parts) was recorded by measurement, the decrease in ventilation would be explainable.

**Airflow caused by a dynamic driver**

The possible influence of wind outside a cave on air movement inside the cave is frequently mentioned by many authors (Geiger, 1966; Cigna, 1968; Tuttle & Stevenson, 1978; de Freitas et al., 1982; Pflichts & Plasecki, 2003; Kowalczyk & Froelich, 2010). Generally perceived as a less usual mechanism, the moving of fluids in both inside (e.g., streams, flood) and outside the cave (e.g., external wind) are considered as a dynamic driver of cave ventilation (Cigna, 1968). However, the outdoor wind as a dynamic driver is often questioned (Christoforou et al., 1996; Russell & MacLean, 2008; Lang & Faimon, 2013). Tuttle & Stevenson (1978) admit its role only in instances of caves with a short simple tunnel between their two or more entrances or shallow caves with a large entrance. On the contrary, Williams & McKay (2015) suppose the external wind to significantly control the balance of cave ice deposits in cases of specific cave morphology. Nachshon et al. (2012) quantified the effect of wind-induced venting of surface fractures within soil and rock environment based on field measurements and laboratory experiments.

No direct link between the external wind and the cave airflow has emerged from the correlation matrix, with the exception of the summer monitoring session. Analysis suggests that the temperature of the flowing cave air $T_{f}$ could be inversely proportional to the outdoor wind $AF_{\text{out}}$ ($r = -0.56$). It means that stronger wind could allow the colder cave air mass to be evicted from the cave. However, it is not clear whether the air from deeper cave levels participates in this ventilation or whether the cave works as a flow heater, warming up the air entering the superficial parts of the cave through the surface cracks. High absolute $R$-values ($0.73$) of the $T_{f} - \Delta T^{*}$ ($T_{\text{out}}^{*}$) relations are a simple consequence of warm air accumulation in the cave entrance. Equally, the $AF_{\text{out}} - \Delta T^{*}$ ($T_{\text{out}}^{*}$) links (absolute $R$-value 0.42) document the dispersion of the warm air mass induced by the increasing speed of outdoor wind.

Testing for variance, performed within two $AF_{\infty}$ datasets differing in speed of wind $AF_{\text{out}}$ seems to be claiming the $AF_{\infty} - AF_{\text{out}}$ connection. The $AF_{\infty}$ set, characterized by non-zero-valued wind velocity ($AF_{\text{out}} > 0$), reflects a significantly higher variance (by more than 110%) than the set documenting the cave airflow under calm wind conditions ($AF_{\text{out}} \sim 0$). This result suggests the external wind to be part of the driving forces of the cave ventilation. However, the $AF_{\text{out}} > 0$ dataset turns out to have a lower mean value of $AF_{\infty}$ by almost 20%, compared with the $AF_{\text{out}} \sim 0$ dataset. It seems to be in contradiction with direct observations in the field, since strong wind conditions intensified water vapor formation near the cave, implying an increased cave airflow velocity. The discrepancy may indicate that intensive cave ventilation does occur through other cracks and vents under strong wind conditions, while the airflow within the entrance parts stagnates. It seems to be a plausible explanation, considering the geomorphic settings of the cave, the surface cracks and numerous relaxed zones identified above the cave (Pánek et al., 2011). However, further study is necessary to confirm this hypothesis.

At any rate, the external wind causes the superficial parts of the cave to be ventilated. It is probably achieved by inducing pressure changes by the wind at the ground-atmosphere boundary. This mechanism is called the Bernoulli effect (Nachshon et al., 2012) and explains the active ventilation regime of the cave in summer. However, a detailed knowledge of how exactly this effect works within the VOC is unclear, since no airflow data on the ventilation of other vents of the cave are available.
CONCLUSION

This work dealt with a complex analysis of the cave airflow and ventilation within the Velká Ondrášova Cave in the Outer Western Carpathians, Czechia. The results of long-term temperature monitoring have shown a strong seasonality of temperature within the near-surface parts of the cave, as an annual temperature amplitude of ~10°C was recorded in the Entrance Dome. Different temperature regimes of the Left and Right Branches of the cave have been explained by different depth, position, and morphology of both parts.

The temperature monitoring is in agreement with the airflow dynamics mapped inside the cave by a chemical tracer. During winter, air mass movements are detectable within almost the whole cave length, fading with the increasing depth of the cave. In summer, all the cave parts are static, except for the shallowest parts of the cave, representing vents within relaxed zones of rock massif and cracks blocked by colluvial sediments.

The cave airflow measurement pointed out an average velocity ranging between 0.27 and 0.61 m s⁻¹ and a maximum velocity of 1.25 m s⁻¹. However, the equivalent average ventilation rate 540–1,260 m³ h⁻¹ corresponding to ~13,000–30,000 m³/day should be taken as a rough estimate of the real value, considering the extensive vent system of the cave determined by the complex cave morphology.

As suggested by statistical testing, ventilation of the superficial cave parts is probably caused by outdoor wind. It induces pressure fluctuations at the ground-atmosphere interface, triggering the ventilation of the shallow cave parts, a mechanism called the Bernoulli effect. However, during winter, the ventilation of deeper cave levels is driven by the temperature gradient, since almost 80% of the analyzed airflow variability has been explained by this predictor within regression analysis.

The model of the Helmholtz resonator appeared to be unsuitable for an explanation of the oscillations occurring on the records of the cave airflow velocity. The analyzed signal of the cave airflow was characterized by multiple frequencies in spectral domain. The cave volume of ~76,000 m³ inferred from the resonance model by applying the highest traced frequency (62.5 mHz/16 s) seems to be meaningless, based on the mapping of the Velká Ondrášova Cave by Wagner et al. (1990). The resonance model failure could have been caused by the unsuitable cave geometry, heavily fractured rock massif, or the influence of external wind on the cave airflow. Confirmed by correlation analysis, repetitive accumulation and wind-induced dispersion of warm air occur in front of the cave entrance, being another possible trigger of the cave airflow oscillations.

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Cave airflow mechanism of a crevice-type cave

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Between worlds: Understanding ritual cave use in later prehistory

Between Worlds’ is the second major publication focusing on the ritual use of caves that appears the last few years after Holly Moyes (Ed.) ‘Sacred Darkness’ in 2012. Generally, the last decade saw a rise on high quality publications on cave archaeology starting with Bergsvik and Skeates ‘Caves in Context’ (2012) and following by the ‘Archaeology of Darkness’ in 2016. What all these previous publications have in common is that they acknowledge caves as a distinct archaeological context – as an enclosed landscape with special environmental conditions – that requires a different approach for its study and interpretation comparing to open air or built archaeological sites. What ‘Between Worlds’ is doing differently – and I much appreciated this – is that in comparison to the previous publications brings the ‘cave’ as natural formation on the forefront of the discussion.

‘Between Worlds’ is structured in two parts that follow a stand-alone introduction from the authors. The introduction summarises really well the current theoretical discussion on cave archaeology and the interpretation of cave use, and presents in a thorough manner the arguments that have been expressed in earlier major publications. Part one has eight chapters that offer strong theoretical discussions and arguments about caves as an anthropological dynamic space entities. In this part, it seems that the discussion that started in 2012 by Mlekuž, that cave is a natural formation, a dynamic enclosed landscape, that offers certain affordances to its visitors reaches a conclusion, particularly with the discussion in the chapters from Peterson, Mlekuž, Prijatelj and Skeates. Generally, the first part of the volume is really robust with a rigorous theoretical discussion in the beginning that leads to four well-presented case studies from Greece, Britain, Italy, and Belgium. I feel that the ‘Theoretical Manifesto’ chapter 2 and the ‘Caves Agency’ chapter 3 in particular they will attract attention in the near future mainly because they stand on coherent arguments about the ‘physicality’ of the caves as both places and spaces that influence – if not shape – human actions.

The second part of the volume has four chapters and ‘explores new ways of investigating dynamic cave environments, with particular focus on digital capture technologies’ (p. 5). This part is innovative, with interesting case studies from Scotland and France, that showcase an array of digital methodologies for the study of cave rituals, but lacks the theoretical breath and strength of the first part, minus the interesting Waller’s chapter on the archaeoacoustic modelling. The volume would be really fascinating if the second part’s methodologies and techniques were in a ‘dialogue’ with the first part’s theoretical perceptions, instead being stand-alone presentations of state-of-the-art digital applications in cave archaeological research.

Overall, as also my feelings for the previous H. Moyes ‘Sacred Darkness’ volume are, I think that ritual cave uses should be studied comparatively with domestic – profane uses of the caves. So as to move in cave use interpretation beyond the Durkheimian sacred/profane dichotomy. It is true to believe that in the symbolically enhanced – with cave decoration, water dripping, mystic air flows etc – cave environments all uses can be ritualised, such as the annual sheep shearing in the Greek cave barns which has perceived almost a as a cult by the shepherds. Nevertheless, without acknowledging the diachronic economic – practical – aspects of cave use as animal pens, storage facilities, shelters, and more, I think we undermine their importance as parts of the societies’ everyday lives.

I believe ‘Between worlds’ is the last stretch of a long way towards the understanding of the role of caves as natural environments have in the archaeological discourse. This volume is the epitome of the theoretical discussion that started in 2012 and for this only it is a valuable read and a significant publication for the archaeological study of
caves. I also believe that the weakness of the volume to address decisively the sacred-profane dichotomy on the interpretation of cave use is not something that downgrade its importance. On the contrary, after the publication of ‘Between Worlds’ cave archaeologists should feel ready to move on to this endeavour and to make it the new frontier.

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