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Stygobitic crustaceans in an anchialine cave with an archeological heritage at Vodeni Rat (Island of Sveti Klement, Hvar, Croatia)

Alessandro Montanari ^{1*}, Nicolas Cerveau ², Barbara Fiasca ³, Jean-François Flot ⁴, Diana Galassi ³, Maurizio Mainiero ⁵, David McGee ⁶, Tadeusz Namiotko ⁷, Stefano Recanatini ⁸, and Fabio Stoch ⁴

¹Osservatorio Geologico di Coldigioco, Cda. Coldigioco 4, 62021 Apiro, Italy

²Department of Geobiology, Georg-August Universität, Goldschmidtstraße 3, 37077 Göttingen, Germany

³Department of Life, Health and Environmental Sciences, University of L'Aquila, Via Vetoio, Coppito, 67100 L'Aquila, Italy

⁴Evolutionary Biology & Ecology, Université Libre de Bruxelles, C.P. 160/12, Avenue F.D. Roosevelt 50, 1050 Brussels, Belgium

⁵Studio Geologico, Via XXIX Settembre 2/O, 60122 Ancona, Italy

⁶Department of Earth, Atmospheric and Planetary Sciences, Massachusetts Institute of Technology, Cambridge, MA 02142, USA

⁷Department of Genetics and Biosystematics, University of Gdansk, Wita Stwosza 59, 80-308 Gdansk, Poland

⁸Gruppo Speleologico Marchigiano, Via Veneto 10, 60122 Ancona, Italy

Abstract: A group of four amphoras found in the anchialine cave of Vodeni Rat in the Croatian island of Sveti Klement, on a rocky ledge at 24 m water depth, indicates that a freshwater source was exploited at the bottom of this karstic pit by islanders and/or passing-by sailors from the late Roman Republican Period to the Early Medieval Period. In other words, prior to the 4th–7th century CE, Vodeni Rat was not an anchialine cave but a Pleistocene karstic pit with a freshwater pool at the bottom. Seawater started to infiltrate this cavity via newly opened fissures in the surrounding limestone after the 4th–7th century CE, as the archeometric age of the youngest intact amphora found down in the pit suggests. At present, the 29-m-deep water body is stratified, with a saline water layer at the bottom, a freshwater layer at the surface, and a transitional brackish water layer in the middle. Our study of the physical and biological characteristics of the cave water column revealed diversified and highly partitioned populations of stygobitic crustaceans including ostracods, copepods, amphipods, and thermosbaenaceans. Some species known to be exclusively endemic of highly saline habitats were collected from the bottom saline layer, whereas others known to be of freshwater origin were found in the upper water layer. This suggests that the freshwater dwellers were already present in this cave prior to the early medieval event that caused seepage of seawater into this karstic pit.

Keywords: Dalmatian underwater speleo-archeology, ostracods, copepods, amphipods, thermosbaenacean
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INTRODUCTION

An anchialine cave with an archeological heritage

On the steep, treacherous rocky littoral of Vodeni Rat (literally “water cape”), in the little Island of Sveti Klement, the largest of the Pakleni Archipelago that festoons the southwestern offshore of the Island of Hvar, Croatia (Fig. 1), there is a small anchialine cave, with a narrow elliptical entrance at about 12.5 m above sea level and some 30 m distance from

the sea. An anchialine cave is broadly defined as “a tidally-influenced subterranean estuary located within crevicular and cavernous karst and volcanic terrains that extends inland to the limit of seawater penetration.” (Bishop et al., 2015). This type of cave often contains a body of saline water at depth, fresh or brackish water at the surface, and a gradient of increasingly saline water in the middle, and it is common among the many known caves along the littorals of the Croatian karst (Radić Rossi & Cukrov, 2017 and references therein).

*sandro.coldigioco@gmail.com

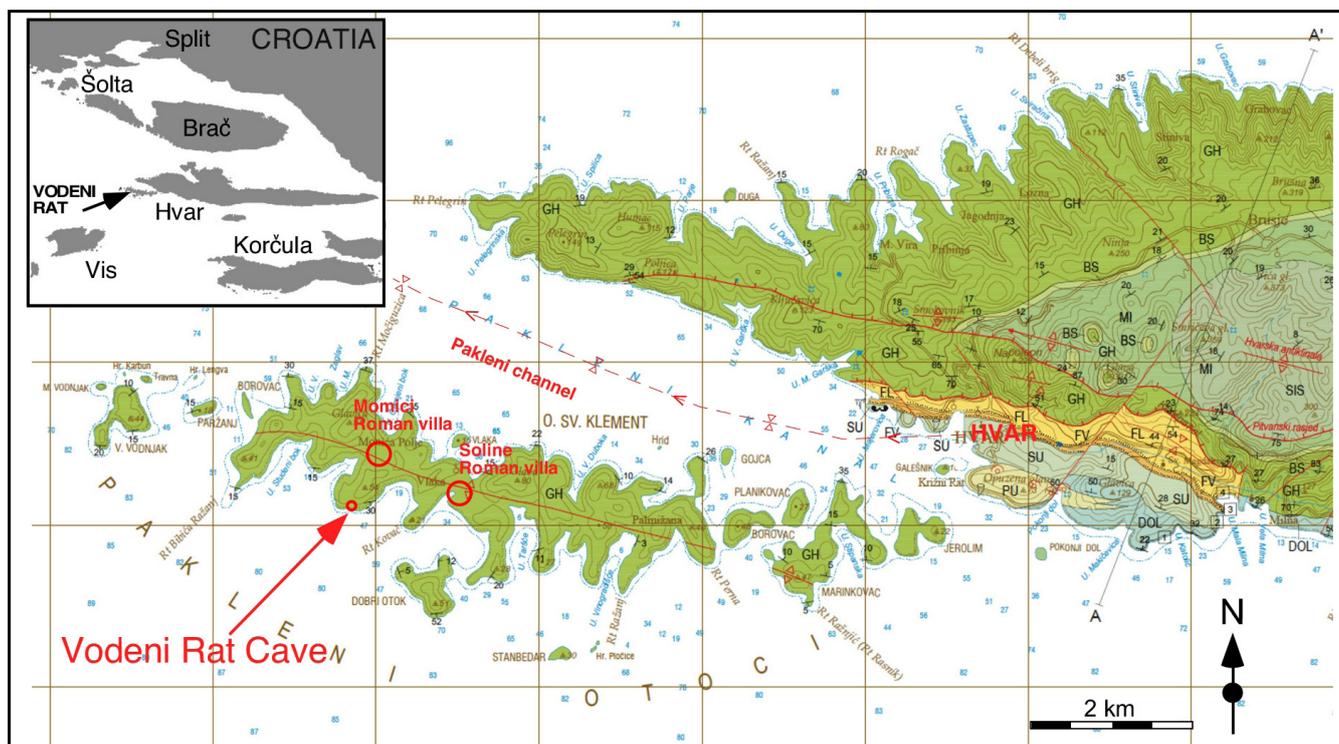


Fig. 1. Location map of Vodeni Rat Cave. Geologic base map from Oštrić et al. (2015).

The cave of Vodeni Rat was first explored in the summer of 1999 by a team of cavers and speleo-scuba divers from the speleoclub Špiljar of the city of Split, during a surveying campaign of karstic caves in the Dalmatian islands in search of potential potable water sources (Mesić, 2006). During that expedition, the speleologists found out that the cave was essentially a narrow, ~13 m deep vertical shaft ending on a water pool, which measured ~1.5-2 m in width, ~6-8 m in length, and ~29 m in depth. The water column was clearly stratified with saline, somewhat turbid water at depth, and fresh clear water at the surface. Despite the scarce visibility, especially at depth in the saline part of the water column, the scuba divers noted the presence of broken ceramic vases and four well-preserved amphoras laying on the side over a rocky ledge at about 24 m water depth (i.e., 24 m below mean sea level). This archeological discovery was immediately reported to the local authority at the Heritage Museum of Hvar, and on December 8 of the same year an expedition was organized and led by archeologist Jasen Mesić, with the main goal of retrieving, classifying, and preserving the archeological finds. In addition to Mesić, nine other people participated in the expedition, including speleologists and scuba divers from the speleoclub Špiljar, archeologist Marinko Petrić from the Heritage Museum of Hvar, and a logistical support team of local people (Petrić, 2017). The difficult operation of recovery of the archeological finds was first described in a report written in Italian by Mesić (2006), in a book chapter in Croatian by Petrić (2017), and ultimately in an abridged account in English by Radić Rossi & Cukrov (2017). The whole operation of recovery, including the dive to the bottom of the cave pool at ~29 m below mean sea level, was filmed and the original videotape is presently preserved at the Heritage Museum of the City of Hvar, along with

the retrieved amphoras now exposed in the adjoining archeological museum.

Cave archeometry

The archeological finds in the cave of Vodeni Rat undoubtedly indicated that in antiquity this freshwater source was exploited for a long period of time. A massive, 95-cm-tall and essentially intact Lamboglia-2 type amphora found on the submerged ledge at 24 m water depth (i.e., 24 m below mean sea level and about 37 m below the entrance of the cave) dates back from the 2nd century BCE to the first half of the 1st century CE, whereas a perfectly preserved 52-cm-tall LR-1 type amphora found next to it (Mesić, 2006; Petrić, 2017) was likely manufactured somewhere in the northeastern Mediterranean region (i.e., Cilicia province of southeastern Turkey, or Cyprus) from the second half of the 4th to the early 7th century CE (e.g., Piéri, 2005; Reynolds, 2005). Both these amphoras were internally coated with still-preserved pitch (Mesić, 2006), indicating that they were meant to contain liquids. According to Mesić (2006), these amphoras were purposely left on the ledge, which was located on the side off the vertical line of the well, and served to scoop up freshwater at the bottom of the 37-m-deep vertical shaft. The precious potable water was probably lifted up and out the well in goat skin water bags (*uter* in Latin), which would not break when banging on the walls of the narrow shaft, and would have been easily transported on donkey back to the Roman *villa rustica* (i.e., a farm) of Momići, some 700 m to the northeast of the cave, or the larger *villa maritima* of Soline, about 1,500 m to the east of the cave (see Fig. 1 for location), both dating to the first half of the 1st century CE (Begović et al., 2013). Probably, the drinking water of Vodeni Rat was also utilized by passing-by sailors who could easily and safely dock in the Uvala Okorisa, a deep

cove constantly protected from winds and waves, and located just 400 m to the north of the cave.

During the expedition of 1999, the speleo-scuba divers also reported crafted wooden beams wedged into the rock walls of the cave presently at 6 and 9 m water depth, which probably represent the remnants of some permanent infrastructure such as a scaffolding that would have facilitated the non-trivial operation of lifting up the water (Mesić, 2006). Speleo-scuba divers also reported abundant raw wood remains at the very bottom of the cave's water pool, which

probably represented torches or possibly pieces of stepladders as parts of the scaffolding. This operation of water lifting certainly needed several people at work, down inside the cave standing on the ledge 37 m below the cave entrance to fill up the water bags (or any other type of unbreakable container such as a wooden bucket), and outside the cave to lift up with ropes the containers filled up with precious drinking water (Fig. 2A).

In fact, the southeastern rim of the cave entrance is incised by four ~1-cm-wide grooves, the longest

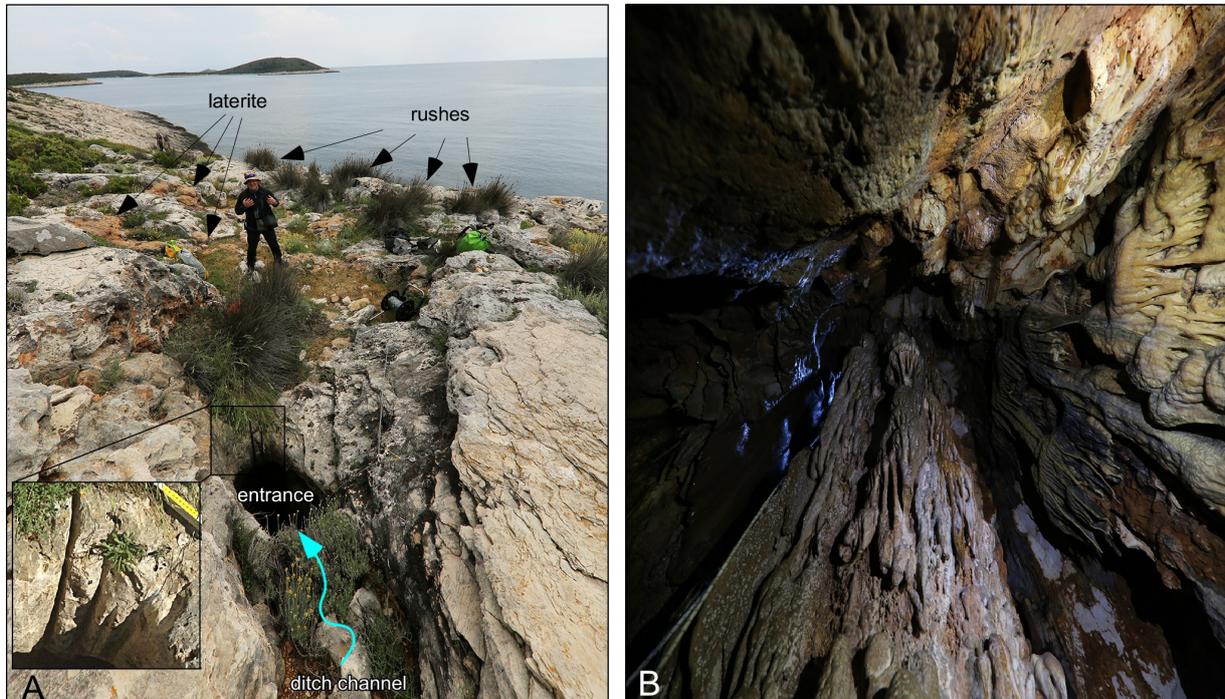


Fig. 2. A) Panoramic view of the Vodeni Rat site. In the distance, the Islet of Dugi Otok. (photo by A. Montanari); B) The speleothem-covered walls of the Vodeni Rat Cave looking up from the “bridge” (see Fig. 6 for location) (photo by S. Recanatini).

of which is about 10 cm deep (see inset in Fig. 2A), which were produced by the abrasive friction of ropes. All this indicates that the operation of uplifting water bags or other containers with ropes by one or more people standing outside the cave entrance has been going on for a long period of time, from the 2nd century BCE to the 4th–7th century CE, as suggested by the typology of the amphoras.

All these archeological and speleologic evidences strongly suggest that until the 4th–7th century CE the cave at Vodeni Rat was not an anchialine cave, i.e., a “tidally-influenced subterranean estuary...” (Bishop et al., 2015), but rather a ~37 m deep karstic crevice with at the bottom a pool of fresh, potable water of probable meteoric origin. Eventually, sometime after the 4th–7th century CE, during the so-called Dark Ages Cold Period (DACP, Helama et al., 2017 and references therein), the Roman villas were abandoned, highly saline water started to infiltrate into the cave through joints and fissures of the surrounding limestone bedrock, and ultimately it mixed up with the cave's freshwater. Water became brackish and its level rose some 24 m up to sea level, flooding up what used to be the dry part of the cave. Nevertheless, a superficial layer of light freshwater persisted floating over the denser saline water. This freshwater layer would have been replenished and maintained by the runoff of

meteoric water channeled in a small natural ditch on the up-slope side of the cave entrance (Fig. 2A). In summary and by analogy, the cave hydrology would be like the one of an *impluvium* (i.e., an underground cistern) connected via a narrow well to an external *compluvium*, i.e., a structure meant to collect and concentrate rainwater through gutters and drainage grates. Of course the cistern has to be waterproof so to prevent seepage. In the case of the Vodeni Rat Cave, the waterproofing was provided by thick speleothemic calcite of voluptuous flowstones and stalactites, which completely coat the inner walls of the cave down to about 23 m below mean sea level (Mesić, 2006; see Fig. 2B). By analogy, this speleothemic coating would be like the pitch, which coats the inner walls of otherwise porous ceramic containers meant to hold liquids. Mesić, (2006) hypothesized that sometime in the Early Medieval Period, “tectonic perturbations” (probably alluding to earthquakes) caused the rupture of this waterproofing speleothemic sealing following fracturing of the encasing limestone bedrock.

Serendipitous discovery of stygobitic crustaceans in the cave of Vodeni Rat

The availability of potable water just 30-m-distance from the sea in a small, dry rocky island like Sveti Klement certainly represents an interesting aspect of

the link between human history and geologic history of this particular place, as it was recounted by Mesić (2006). On October 7, 2015, after visiting the ruins of the Roman villa at Soline, and studying the pedology of the adjoined 6 ha *ager* (60,000 m²), i.e., the Roman farmland, we went to Vodeni Rat to check the cave and the surrounding environment. We wanted to try the “bottle test”, which entailed lowering a 1.5 liter clear plastic bottle with a ballast weight attached at the end of a line down the 13-m-deep shaft until getting it completely full of water, finding it agreeably sweet and drinkable. However, we noticed a white animal, about 1 cm long, swimming in the water bottle. The animal, which at first glance appeared to be an amphipod from the way it swam “belly-up”, was extracted from the bottle and fixed in 95% ethanol, and later on it was first sent to one of us at the Georg-August-Universität in Göttingen, Germany for a first identification, and then to one of us at the Université libre de Bruxelles for DNA sequencing.

On June 7, 2017, we returned to the cave of Vodeni Rat, this time armed with a closing Nansen net with a 50-ml Falcon tube as catcher, with which we caught a number of meiofauna organisms by blindly lowering it down to the freshwater pool with a line. A first study of the collected specimens was carried out at the universities of L'Aquila and Brussels (Fig. 3).

In the expedition of May 2018, we collected more samples with the Nansen net and also with a Cvetkov net specifically to collect faunal samples at various

depths in the water column. In addition, we carried out a thorough, detailed survey of the environment surrounding the cave. The upper dry part of the cave was also inspected and sketched, whereas the water column was analyzed for temperature, pH, electric conductivity, and salinity with an immersion probe down to 21.5 m water depth.

In this paper, we describe in detail the geological, geomorphological, and biological characteristics of the environment surrounding the cave of Vodeni Rat, the physical properties of the water column of the saturated part of the cave, the systematics and ecology of the collected stygobitic crustaceans, and will discuss about the timing of the crustacean colonization of this cave on the basis of geologic, geochronologic, and archeometric evidences.

GEOLOGIC AND ENVIRONMENTAL SETTINGS

The small Pakleni archipelago represents one of a series of WNW-ESE trending anticlinoria making up the great islands of the central Dalmatian archipelago such as Korčula (the most external), Hvar, Brač, and Šolta (see inset of Fig. 1). In turn, these structures belong to the late Paleogene external Dinaride orogenic complex (e.g., Korbar, 2009; Surić et al., 2014 and references therein). These islands are all made up of limestones of the lower Cretaceous to lower Paleogene succession of the Adriatic Carbonate Platform (ACP in Korbar et al., 2010, 2015; Oštrić et al., 2015 and references therein). The Campanian Gornji Humac Formation (84–72 Ma) is the one exposed at Vodeni Rat (Fig. 1), and represents a succession of medium-to-thick bedded hard limestones including pelletal foraminiferal wackestones, rudist flowstones, and cryptoalgal laminites (Oštrić et al., 2015), with a southern dip of ~30°. The location of the cave entrance can be spotted from the off-shore distance on the steep, barren supralittoral zone of the island just below the edge of the tree line at the western end of an unusually extensive *Juncus acutus* grove, a salt-tolerant rush plant, which is normally very rare in dry, rocky environments such as the karstic littorals of the Dalmatian islands (e.g., Pandža & Milović, 2015; see Fig. 4A). A grove of less conspicuous *Juncus maritimus* rushes is spread on the rocky area to the west of the cave entrance but it is too seemingly out of place inasmuch this rush species is endemic of sandy, salty, and humid habitats (Fig. 4B).

The limestone beds exposed at Vodeni Rat are pervasively crisscrossed by systems of pressure-solution cleavage and joints with various orientations (Fig. 4B). Two sub-vertical ~NNE-SSW-striking minor faults of undetermined offset delimit the area where most of the *Juncus* rush plants are concentrated. A part from the

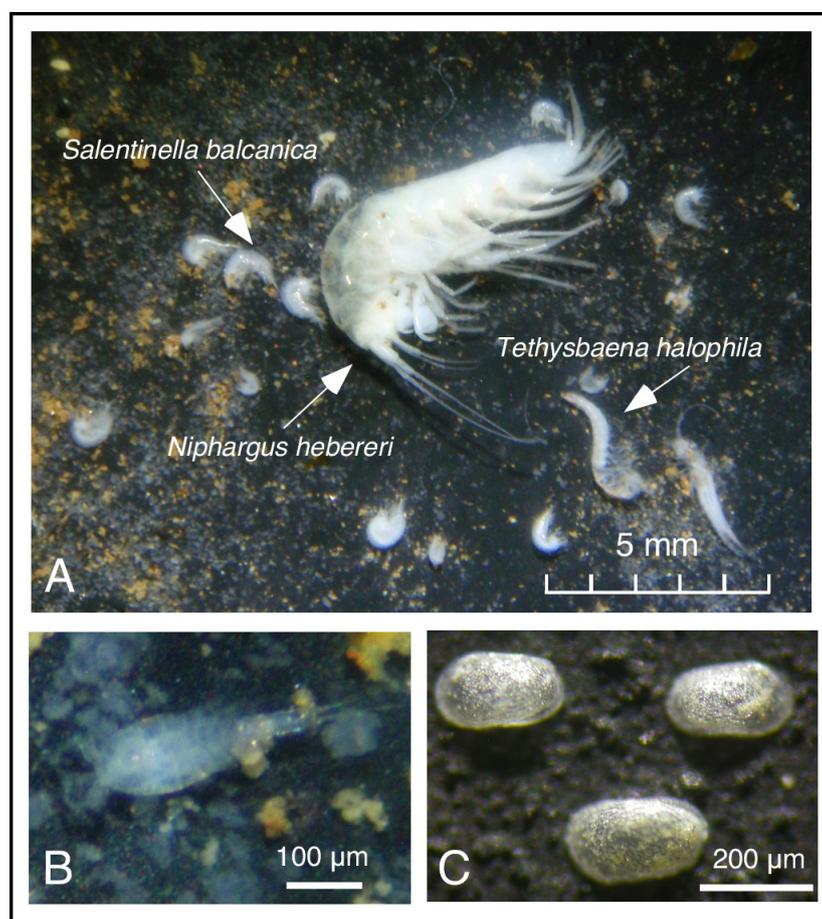


Fig. 3. First reconnaissance of crustaceans in the catch of the day at Vodeni Rat on June 2017 (Zeiss dissecting stereomicroscope photographs by A. Montanari). A) Amphipods and thermosbaenaceans; B) The copepod *Halicyclops dalmatinus* (male); C) Valves of the ostracod *Pseudolimnocythere* sp.

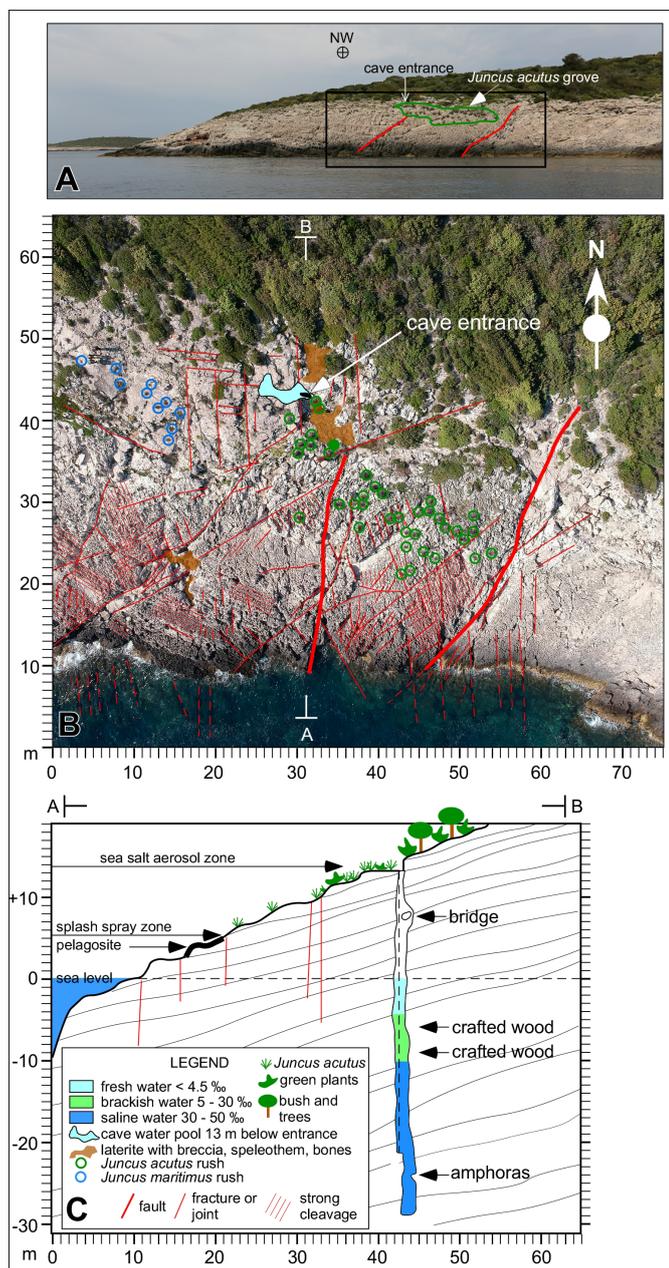


Fig. 4. A) Panoramic view of Vodeni Rat as seen from offshore (photo by A. Montanari); B) Structural setting of the Vodeni Rat site as mapped on a drone orthophotograph (photo by M. Mainiero); C) Cross section and physiography of the Vodeni Rat littoral zone.

Juncus groves, the environmental physiography of the supralittoral zone at Vodeni Rat (Fig. 4C) reflects well the model of Montanari et al. (2019), which was originally defined at Point Pleturina on the southern littoral of the Island of Hvar, some 3 km east from the homonym city. In the immediate surrounding of the cave entrance, the limestone does not exhibit any particularly severe fracturing or jointing, nor there seems to be a ~E-W-striking vertical fault onto which the ~42-m-deep karstic crevasse with a roughly E-W elongation has developed. On the other hand, around the cave entrance there are conspicuous deposits of laterite (Fig. 2B). Horizons and intrusions of laterite and bauxite are commonly found in the mid Eocene limestone succession of the ACP, which indicate a paleokarst underlying a subaerial surface that represents a well-known regional unconformity (Brllek et al., 2014). By contrast, the laterite deposit around the cave of Vodeni Rat is a paleokarst feature

attributable to the Pleistocene karstification of the Dalmatian limestones once they were deformed, uplifted, and exposed following the early Neogene orogenic phase of the external Dinarides (Korbar, 2009). These irregular rust-orange rock bodies are found everywhere around the Pakleni islands as well as in different limestone formations throughout the Hvar main island, and they all seem to fill up cavities or cracks within the karstified limestone. They often comprise limestone breccia and animal bones, but also speleothemic concretions and inclusions of blocky calcite (Fig. 5).

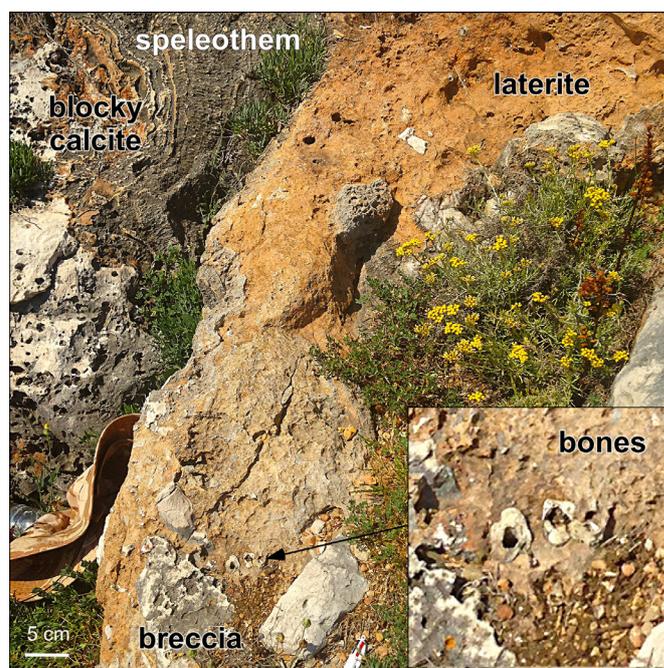


Fig. 5. Close up image of the laterite deposit around the cave of Vodeni Rat (photo by A. Montanari).

A sample of blocky calcite from the Vodeni Rat laterite was dated at the Massachusetts Institute of Technology (USA) with the U-Th method (see Montanari et al., 2020, for dating method and technique), and yielded an age of 302.5 ± 6.3 ka (Table 1) indicating that karstic processes and speleothemic concretion were occurring here in the late Middle Pleistocene. This date suggests that the surface topography of the Pakleni islands did not change much since the Middle Pleistocene. However, the Pakleni channel that separates the archipelago from the island of Hvar (Fig. 1), is likely a synformal structure between the Hvar and the Pakleni anticlinoria, with a NW plunging axis that deepens from the harbor of Hvar to about 70 m below mean sea level at the northwestern end of the channel. This structural setting suggests that the Pakleni channel may represent a now submerged synclinal fluvial valley, which was incised following the orogenic emersion of the external Dinaride.

During the Last Glacial Maximum (LGM) some 25 thousand years ago, the physiography of the Dalmatian islands was very different from the present. For one thing, sea level was about 130 m lower than it is today and the Adriatic coast line was some 30 km to the south of Vodeni Rat. Practically, the islands of Brač, Hvar, and Korčula were rocky hills separated by the now submerged valleys of the Cetina and Neretva

Table 1. Results from U-TH analysis of speleothemic blocky calcite from Vodeni Rat (Islet of Sveti Klement, Hvar, Croatia)

MIT McGee Lab	Sample	^{238}U	\pm (2σ)	^{232}Th	\pm (2σ)	$\delta^{234}\text{U}$	\pm (2σ)	$(^{230}\text{Th}/^{238}\text{U})$	\pm (2σ)	$^{230}\text{Th}/^{232}\text{Th}$	\pm (2σ)	Age (yr)	\pm (2σ)	$\delta^{234}\text{U}$ initial	\pm (2σ)	Age (yr BP) ^f	\pm (2σ)
sample ID	description	(ng/g) ^a		(pg/g) ^a		(per mil) ^b		activity		ppm atomic		(uncorrected) ^c		(per mil) ^d		(corrected) ^e	
VDR-CAL	blocky calcite	47	1	4488	90	28	2	0.9740	0.0027	160	0.3	305300	6100	66	5	302400	6300

^aReported errors for ^{238}U and ^{232}Th concentrations are estimated to be $\pm 1\%$ due to uncertainties in spike concentration; analytical uncertainties are smaller;

^b $\delta^{234}\text{U} = ((^{234}\text{U}/^{238}\text{U})_{\text{activity}} - 1) \times 1000$;

^c $(^{230}\text{Th}/^{238}\text{U})_{\text{activity}} = 1 - e^{-\lambda^{230}\text{T}} + (\delta^{234}\text{U}_{\text{measured}}/1000)[\lambda^{230}/(\lambda^{230} - \lambda^{234})](1 - e^{-(\lambda^{230} - \lambda^{234})\text{T}})$, where T is the age. "Uncorrected" indicates that no correction has been made for initial ^{230}Th ;

^d $\delta^{234}\text{U}_{\text{initial}}$ corrected was calculated based on ^{230}Th age (T), i.e., $\delta^{234}\text{U}_{\text{initial}} = \delta^{234}\text{U}_{\text{measured}} \times e^{(\lambda^{234} - \lambda^{230})\text{T}}$, and T is corrected age;

^eAges are corrected for detrital ^{230}Th assuming an initial $^{230}\text{Th}/^{232}\text{Th}$ of $(4.4 \pm 2.2) \times 10^{-6}$;

^fBP stands for "Before Present" where the "Present" is defined as the January 1, 1950 C.E.;

Decay constants for ^{230}Th and ^{234}U are from Cheng et al. (2013); decay constant for ^{238}U is $1.55125 \times 10^{-10} \text{ yr}^{-1}$ (Jaffey et al., 1971).

rives (Pavelić et al., 2011; Pavelić et al., 2014; Wacha et al., 2019). Aeolian sands deflated from the then dry Adriatic seafloor were blown over the exposed sides of the most external (western) of these mountainous reliefs, such as the present island of Vis (see Fig. 1 for location) by the southerly Jugo wind, building up conspicuous sand deposits (Wacha et al., 2019). The Soline *ager* is, in fact, a rare case of arable land in the arid karstic environment of the Dalmatian islands exactly because its fertile and water-retaining soil is derived from one of these LGM aeolian sand deposits.

Vertical GPS velocities in selected locations in northwestern Croatia indicate that the internal Dinaride is presently uplifting whereas the external Dinaride is subsiding (Surić et al., 2014). A part from some localized cases where emerged tidal notches suggest a slight uplift (e.g., the Dubrovnik coast; Surić et al., 2014), which surpassed the generalized tectonic subsidence of the external Dinaride domain, geomorphic features such as sublittoral caves and other karstic features found below sea level, submerged tidal notches, and supratidal pelagosome encrustation (Surić & Juračić, 2010; Montanari et al., 2019) they all indicate that coastal caves such as the one at Vodeni Rat represent an ancient relict karst, which developed in a continental environment at much higher elevations during the Pleistocene, and ultimately reached sea level or drowned below it following of a slow, generalized post-orogenic subsidence.

SAMPLING

Biological samplings were done by lowering, with a 6 mm nylon line, a Nansen closing net with a 30-cm-diameter iron rim and a 50 ml Falcon tube as a catcher, from the rim of the cave entrance down until touching the surface of the water pool (Fig. 6).

The net was then dipped a meter or so into the water and slowly lifted up and out in the air to let the water filling up the net to filter out through the 44 mm polyester mesh. Vertical hauls were repeated 20 times in free water (sample FREE in Fig. 6). The same technique was used to collect samples along the submerged side of the cave by having the rim of the net brushing against the rocky wall of the pool (sample WALL in Fig. 6). The catch was immediately fixed on site with 95% ethanol, and the Falcon tube properly labeled, sealed with electrical tape, and made

it ready to be sent to the biolab at the University of L'Aquila. In the last expedition to Vodeni Rat we used a phreatobiological Cvetkov net (Cvetkov, 1968) of 60 μm mesh size to collect at mid depths in the water column and also all the way down to about 21.5 m depth in the well, which was the maximum depth we could reach with our blind probing from the entrance of the cave (samples DEEP and BOTTOM in Fig. 6).

The physical properties of the water column including temperature, pH, electric conductivity, and salinity, were analyzed with an YSI 600XLM immersion probe in continuity from 21.5 m water depth (maximum depth reachable with the probe from the rim of the entrance), up to water surface (Table 2).

The probe was attached at the end of a 5 mm steel chain with 50 cm markings and a weight to maintain

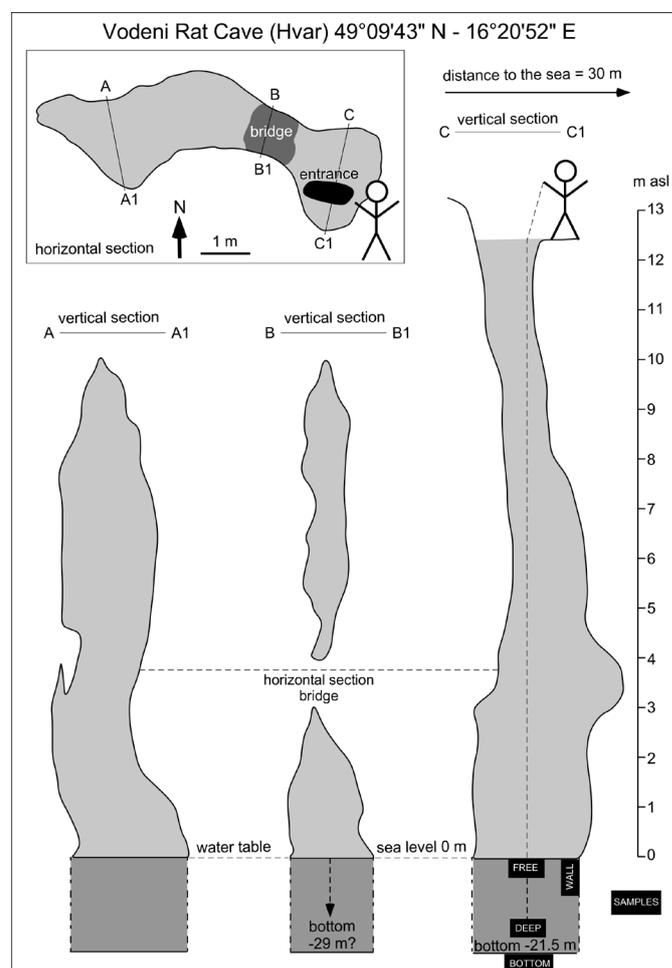


Fig. 6. Sketched horizontal and vertical sections of the cave of Vodeni Rat, and indication of the sampled underwater zones.

Table 2. Immersion probe data from Vodeni Rat Cave (Croatia)

Water depth (m)	Temperature (°C)	pH	Conduct. (µS/cm)	Salinity (‰)
0.985	17.59	7.80	3297	1.74
1.801	17.68	7.80	3758	1.99
2.889	17.73	7.83	4187	2.23
3.699	17.74	7.83	4668	2.51
4.413	17.74	7.77	6097	3.33
5.195	17.72	7.61	8259	4.61
6.089	17.70	7.43	15341	8.98
6.765	17.69	7.36	21385	12.88
7.545	17.66	7.27	26573	16.32
8.219	17.63	7.19	31578	19.72
8.975	17.61	7.14	41222	26.46
9.556	17.60	7.14	44834	29.05
10.122	17.57	7.11	48254	31.53
10.801	17.53	7.07	53463	35.36
11.556	17.48	7.05	57360	38.26
12.542	17.44	7.03	57856	38.64
13.895	17.40	7.03	57984	38.73
14.606	17.38	7.02	58057	38.79
15.562	17.35	7.02	58130	38.84
16.364	17.33	7.01	58157	38.86
17.417	17.31	7.01	58180	38.88
18.290	17.29	7.01	58195	38.89
19.188	17.26	7.00	58220	38.91
20.018	17.25	7.00	58242	38.92
20.881	17.22	7.00	58278	38.95
21.496	17.20	6.99	58310	38.97

Measurements done on 09/05/2018 from 14:48 to 14:50 hrs.

it in tension and vertical in the water column. While temperature from 17.60°C at the surface increases by 0.15°C through the top 4 m of the water column to then gradually descend through the rest of the water column down to 17.20°C at 21.5 m water depth, all the other parameters indicate a well stratified water column with the top 4 m made up of freshwater (salinity = <5‰), followed by a 4.5-m-thick mixing zone of brackish water (salinity = 5-30‰), and a bottom layer of essentially sea water (salinity = ~40‰) (Fig. 7).

SYSTEMATICS AND ECOLOGY OF STYGOBITIC CRUSTACEANS IN THE CAVE OF VODENI RAT

Ostracods

From the WALL catch of June 2017, we hand picked 25 well-preserved single valves of a tiny ostracod that we identified as *Pseudolimnocythere* sp. (Figs. 3C and 8).

Unfortunately, no soft parts nor paired and closed articulated valves were found in that batch, and so it was impossible to identify the actual species. Nevertheless, this genus is very interesting inasmuch it belongs to the family of *Loxoconchidae* Sars, 1925, which consists mostly of marine or brackish water species. The only other genera of this family, which occur in fresh or brackish waters, are *Cytheromorpha* Hirschmann, 1909 (some species in Europe) and *Sanyuania* in Thailand. There are only two known

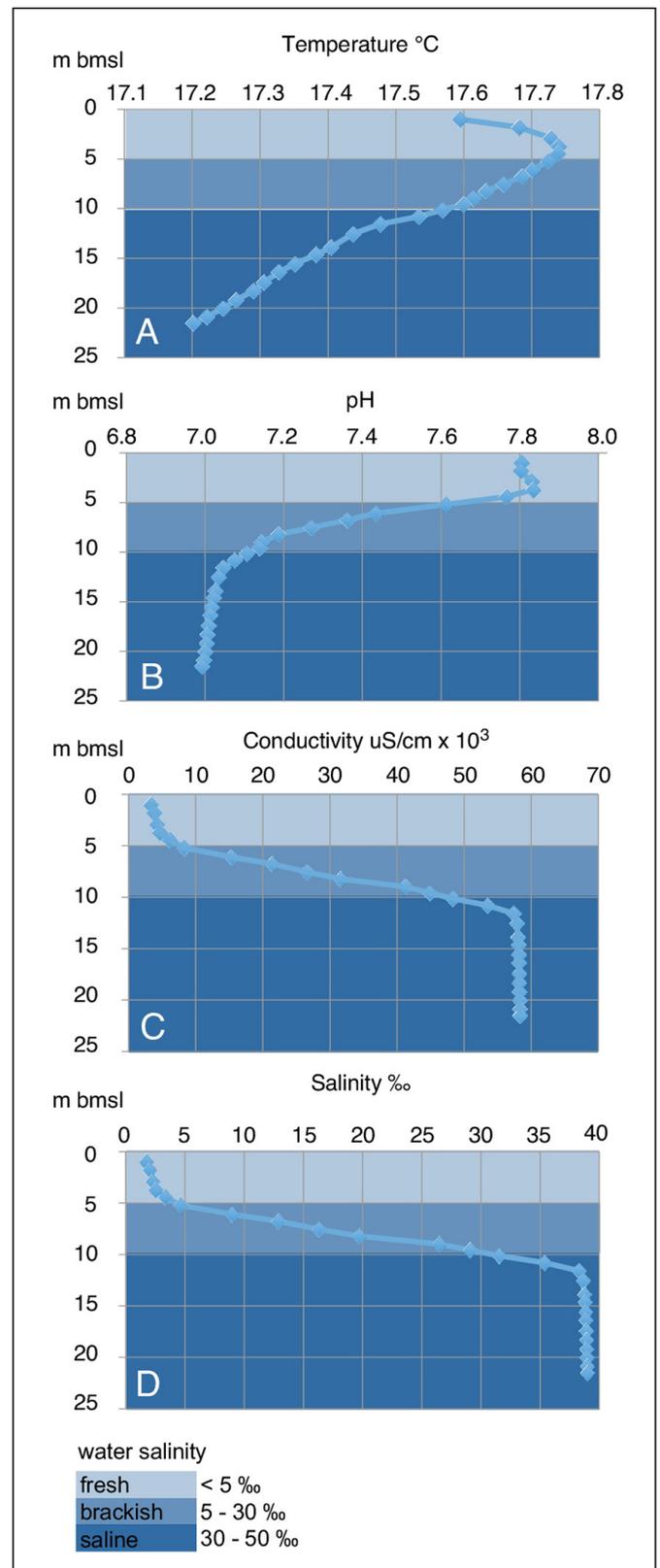


Fig. 7. Profiles of A) temperature; B) pH; C) electrical conductivity; D) temperature, through the water column of the Vodeni Rat cave (see raw data in Table 2).

recent species belonging to *Pseudolimnocythere* Klie, 1938, one of which seems to be endemic to southern Italy - *Pseudolimnocythere hypogaea* described by German ostracodologist Klie (1938) from wells in Bari, and resampled by Karanović & Pesce (2001) from underground freshwaters in Apulia (southeastern Italy). The second recent species of the genus is *Pseudolimnocythere hartmanni* described from wells and interstitial habitats of Euboea Island (Greece)

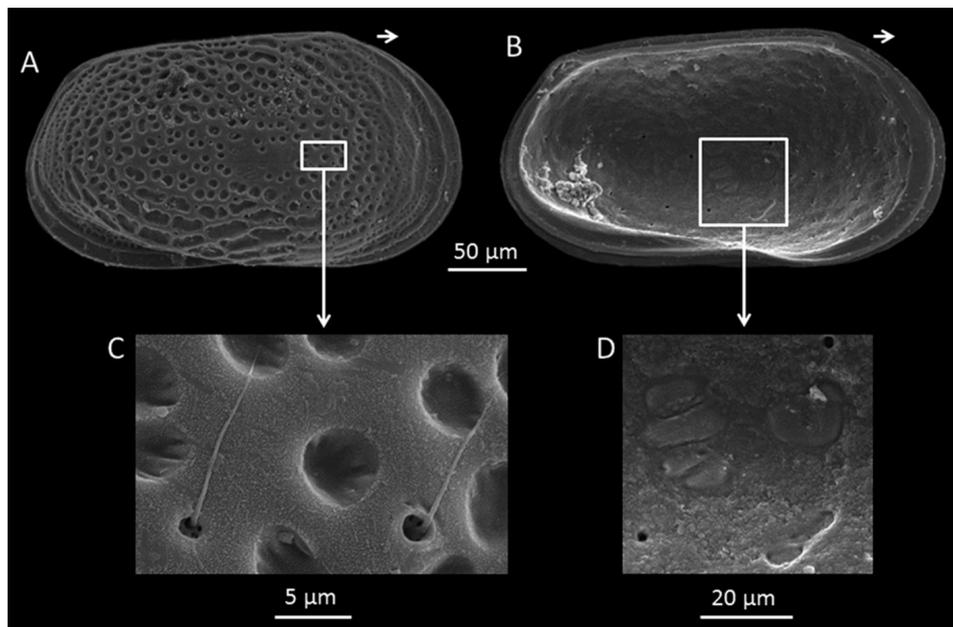


Fig. 8. SEM images of ostracod *Pseudolimnocythere* sp. from the cave of Vodeni Rat. A) Right valve external view (length = 278 µm); B) Left valve internal view (length = 281 µm); C) Detail of ornamentation and two sieve pores; D) Detail of muscle scars (photos by T. Namiokto).

by Danielopol (1979). There are some more recent/subfossil records of *Pseudolimnocythere* from Italy, as a spring near Parma and karst springs in the Secchia River valley in the northeastern Apennines (Pieri et al., 2015 and references therein). Peterson et al. (2013) found over a thousand valves of *Pseudolimnocythere* sp. in phreatic waters of the Frasassi cave complex but only a few of them in the adjacent Sentino River. All these findings are left in open nomenclature as *Pseudolimnocythere* sp., being so small, is extremely difficult to identify taxonomically from just the valves. There is also one fossil species *Pseudolimnocythere hainburgensis* described by Danielopol et al. (1991) from Miocene fresh or brackish water deposits of the Vienna Basin. Very interestingly, Danielopol (1980) in a paper on the origin of European interstitial freshwater ostracods mentioned and illustrated one more species of *Pseudolimnocythere* (also left in open nomenclature *Pseudolimnocythere* sp.) from the Škuljica Cave, near a lighthouse in the southeastern tip of the Krk Island, some 400 km north from Vodeni Rat along the Croatian shore of the Adriatic Sea. Supposing that ancestors of *Pseudolimnocythere* were originally marine species, Danielopol (1980) proposed two hypotheses of how representatives of this genus became continental hypogean species. Hypothesis 1: the *Pseudolimnocythere* species penetrated into the inland subterranean waters during one of the many Pleistocene regressive-transgressive phases. Hypothesis 2: the *Pseudolimnocythere* species penetrated into the inland subterranean waters during the Upper Miocene - Lower Pliocene regressive phase (e.g., during the Messinian salinity crisis).

Danielopol's hypotheses are interesting and relevant for the Vodeni Rat neo-anchialine cave, and respond well to what Bonacci (2009) calls "Karst Ecohydrology", an extremely complex generalized model. But the issue becomes more complicated by the fact that submersion or emersion of karstic systems, which in any case can only form in subaerial regions, also

depend on local tectonic movements, which can be uplift or subsidence regardless of eustatic sea level changes, and they may change or even reverse through geologic time. For instance, the Island of Krk where the Škuljica Cave contains *Pseudolimnocythere* sp. is presently uplifting at GPS velocities of about 4 mm/yr (Surić et al., 2014). So during the LGM about 25 ky ago, when sea level was 130 m lower than today, the Škuljica Cave was located about 30 m above sea level but during the last interglacial (the Eemian or MIS 5e), some 100 ky ago, when sea level was about the same as today, the cave must have been located 400 m below sea level. But this gross calculation with the present GPS velocity does not work (probably because of their large analytical error) inasmuch submerged speleothems in the U Vode anchialine cave in Krk Island at 13–18 m below mean sea level indicated that they were uplifted for approximately 13–18 m during the last ~75–85 ka, so at an average velocity for that period of ~0.15–0.25 mm/yr. Radic Rossi & Cukrov (2017) summarized that "...The chronological determinations of these and 12 other speleothems from five different caves (taken up to a depth of 41.5 m) were realized with three different dating techniques. The results allowed Surić et al. (2014) to reconstruct a partial sea-level curve for the Croatian part of the Eastern Adriatic coast for the last 220,000 years (Surić & Juračić, 2010), and to suggest the dynamics associated with the palaeoenvironmental changes" since the late Middle Pleistocene.

On the other hand, the anchialine caves on the western coast of the Istrian peninsula, which is presently subsiding at GPS velocities of about 4 mm/yr, would have had a different history. Practically, they should have been well above sea level all the time during the Pleistocene, of course if subsidence was going on uninterrupted during the whole Epoch. This is because the Istrian Peninsula along with the central Dalmatian Archipelago (so including the island of Sveti Klement) belong to the external Dinaride tectono-

stratigraphic unit, which is in a generalized regime of subsidence, as opposed to the internal Dinaride unit, which includes the Island of Krk and the northern Dalmatian archipelago (e.g., Kvarnar, Kornati) as well as the Dinaric fold and thrust mountain belt as a whole, which are presently in a generalized regime of tectonic uplift. Going back to the primordial marine origin of the *Pseudolimnocythere* sp., it could well be that both hypotheses 1) and 2) of Danielopol (1980) are right to some extent, in the sense that since the Miocene at least, this marine ostracod lineage (as it seems to be in the fossil record of the Vienna Basin) may have penetrated into the inland subterranean waters from the sea at different times in different places (such as Frasassi, Euboea, Krk, Apulia, Parma, Secchia valley, and Vodeni Rat), and in each case, it became a different species (or possibly there were different species in the beginning within different parts of the western Tethyan or Paratethyan

seas). Only *P. hartmanni* and *P. hypogea*, both with soft parts, were formally described so far as different recent freshwater species.

Copepods

Biological samples were taken using a phreatobiological Cvetkov net, which allowed the collection of fauna at different water depths down to the bottom of the cave. Samples preserved in 95% ethanol were sorted under a Leica M205C microscope and copepods were identified to the species level using a Leica DM2500 optical microscope. A total of 73 copepod specimens were collected. Two of them were assigned to the order Calanoida, being specimens at a very early stage of development and not well preserved thus preventing a finer taxonomic identification. Four copepod species were identified belonging to 3 different orders: Misophrioida (1 species), Cyclopoida (2 species) and Harpacticoida (1 species) (Table 3).

Table 3. Crustacea Copepoda from the cave of Vodeni Rat (Croatia).

Taxon	Number of specimens per catch sample name and relative water depth in m below mean sea level and salinity (parts per mil)			
	FREE 1 m bmsl	WALL 1 m bmsl	DEEP 14 m bmsl	BOTTOM 21.5 m bmsl
<i>Speleophria mestrovi</i>	0	0	28	2
Calanoida indeterminate	0	0	2	0
<i>Halicyclops dalmatinus</i>	2	35	0	0
<i>Diacyclops slovenicus</i>	0	2	0	0
<i>Parapseudoleptomesochra</i> sp.	0	2	0	0
	1.7 ‰		38.7-39.0 ‰	

All the species are strictly stygobites and commonly found in anchialine caves. In the cave of Vodeni Rat, they showed high habitat partitioning, with the misophrioid *Speleophria mestrovi* Kršinic, 2008 exclusively found in samples from the deepest part of the cave and near its bottom where the highest salinity values were measured. In the most superficial layers near the cave walls, 3 different species coexisted in a freshwater habitat (salinity <2‰; Table 2). The dominant species was the cyclopoid *Halicyclops dalmatinus* Petkovski, 1955 (Fig. 3B). It belongs to the cosmopolitan genus *Halicyclops* Norman, 1903 of the family *Halicyclopidae* Kiefer, 1927 (Khodami et al., 2019). The genus includes planktonic and benthic species widely distributed in several kinds of coastal brackish water bodies among which many anchialine caves and sinkholes, but also in freshwater habitats. Before the present record, *H. dalmatinus* was documented in only a few brackish water bodies of Montenegro and in a single brackish water well near Bari (southeastern Italy) (Petkovski, 1955; Pesce et al., 1984). The cyclopoid *Diacyclops slovenicus* Petkovski, 1954 was originally described from the Vrhnika karstic spring of Ljubljana (Slovenia) and subsequently found in several other localities in Slovenia, Croatia, and Bosnia and Herzegovina (Brancelj, 1987; 1990; 2001; Culver & Sket, 2000; Petkovski, 1954; 1984). The species is typical of the saturated karst and was found in the freshwater layer of the cave.

From a taxonomic perspective, *Speleophria* Boxshall & Iliffe, 1986 is a very poorly diversified genus currently comprising six species, namely, *S. bivexilla* Boxshall & Iliffe, 1986 (Bermuda); *S. bunderae* Jaume, Boxshall & Humphreys, 2001 (Cape Range Peninsula, Australia); *S. germanyanzezi* Suárez-Morales, Cervantes-Martínez, Gutiérrez-Aguirre & Iliffe, 2017 (Yucatán Peninsula, Mexico); *S. gymnesica* Jaume & Boxshall, 1996 (Mallorca Island, Spain); *S. mestrovi* (Vis Island; Croatia); *S. nullarborensis* Karanovic & Eberhard, 2009 (Nullarbor Region, South-Western Australia).

All the species but *S. mestrovi* show a point geographic distribution, being at present known from a single cave and can be considered narrow endemics. On the contrary, *S. mestrovi*, originally described from Supurina Cave in Vis Island, some 30 km SW of the Pakleni archipelago (Kršinic, 2008), is now recorded in the cave of Vodeni Rat in Sveti Klement Island during the present investigation.

Members of the genus *Speleophria* have a disjunct distribution. The species are spread out from the Caribbean area to the Mediterranean area and Australia, thus supporting a very ancient origin of the genus. The present distribution of *Speleophria* offers a good example of the so-called full 'Tethyan track' (Croizat, 1958). It covers the area flooded by the Tethys Ocean and by the seas originated by the Tethys fragmentation in the late Mesozoic

(Galassi et al., 1999; Galassi, 2001, Humphreys, 2004). The ancestor of the genus *Speleophria*, distributed in a suitable habitat along the coastlines of the Tethys realm, probably underwent allopatric speciation by vicariance, in which case the species of the genus *Speleophria* can be considered Tethyan relicts.

From an ecological perspective, copepod assemblages in the cave of Vodeni Rat parallel those observed in many anchialine caves around the world composed of calanoids, halicyclopid cyclopoids, and speleophriid misophrioids. It is, indeed, widely recognized that anchialine caves host diverse crustacean assemblages with highly predictable taxonomic composition. Jaume et al. (2001) highlighted the recurrent composition of the communities in anchialine habitats as composed of atyid shrimps, thermosbaenaceans, hadziid amphipods, cirrolanid isopods, remipeds, thaumatocypridid ostracods, and a vast array of copepods such as epacteriscid, pseudocyclopiid, and ridgewayiid calanoids, halicyclopid cyclopoids, speleophriid misophrioids, and superornatiremid harpacticoids. This observation suggests that the crustacean diversity of the cave of Vodeni Rat is probably underestimated and further sampling campaigns are needed to fully figure out the composition of the stygobitic communities. Moreover, additional samples will be needed for precise identification of the unique female individual of the ameirid harpacticoid *Parapseudoleptomesochra* sp. that was collected in Vodeni Rat.

Amphipods

Amphipods are among the most common and widespread crustaceans in Dinaric anchialine caves (Sket, 1999; Gottstein et al., 2012), where they are represented by three families, namely, *Hadziidae* Karaman, 1943, *Salentinellidae* Bousfield, 1977, and *Niphargidae* Bousfield, 1977. *Hadziidae*, although widespread along the whole Dalmatian coast (Sket, 1986; in Kornati Islands they were present in 42% of the caves: Gottstein et al., 2007), have so far not been recorded in Vodeni Rat, whereas the other two families are each represented in the cave by only one species.

The family *Salentinellidae* comprises small (up to 3 mm long) stygobitic amphipods of uncertain affinities inhabiting both freshwater and anchialine caves and interstitial waters, widely distributed in the Mediterranean basin and along the Dalmatian coast (Sket, 1969, 1986). *Salentinella balcanica* Karaman, 1953 (Fig. 3A) was described from a cave near Dubrovnik (as *S. gracillima balcanica*, *S. gracillima* Ruffo, 1947 being a species known from Apulia). It was subsequently synonymised with *Salentinella angelieri* Delamare Deboutteville & Ruffo, 1952 from Corsica by Karaman (1979). However, a research in progress at the Université libre de Bruxelles on the phylogeny of the genus using molecular methods indicates that Dalmatian and Apulian populations are closely related each other, but show no affinities with the Corsican *S. angelieri*. For this reason, pending a nomenclatorial revision, in this study we maintain the binomial

Salentinella balcanica for Dalmatian populations. Although widely distributed along mainland Dalmatia (Sket, 1969, 1986), the species seems very rare in island caves, having been found so far only in an anchialine cave on the islet of Gangaro, Kornati archipelago (Karaman & Sket, 1990). Unexpectedly, this species was the most common malacostracan collected in the cave of Vodeni Rat.

The family *Niphargidae* includes more than 400 described species, mainly stygobitic, distributed in the Western Palearctic. *Niphargus hebereri* Schellenberg, 1933 found in Vodeni Rat (Figs. 3A and 9A) is the most widespread crustacean species in anchialine caves along the Dalmatian coast (Gottstein et al., 2012); in Kornati islands a study found it in 83% of the sampled caves (Gottstein et al., 2007). Although on the nearby island of Hvar the most common species is *Niphargus hwarensis* Karaman, 1952, *N. hebereri* was recently collected in the island of Brač (Rađa et al., 2020).

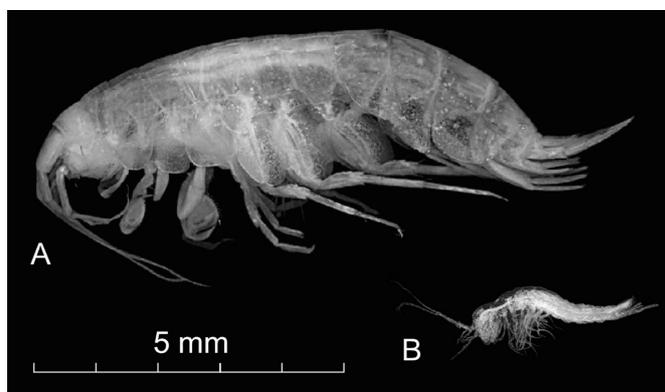


Fig. 9. A) The amphipod *Niphargus hebereri*, and B) the thermosbaenacean *Tethysbaena halophila*. Microphotographs taken under a stereomicroscope Leica M205C by B. Fiasca.

Described from a cave in the Istrian peninsula, it is found in fresh and brackish water caves along the coast as well as on islands; Sket (1966) hypothesized that it is a 'limnic immigrant' that tolerates mixohaline waters. This hypothesis was confirmed in a recent paper on the phylogeny of transadriatic *Niphargidae* (Delić et al., 2020). According to the latter study, during the Messinian Salinity Crisis, i.e., approximately 5.9–5.3 Ma, the ancestor of *N. hebereri* likely spread from the Apennine peninsula (where closely related species such as *N. ictus* Karaman, 1985 from the Frasassi cave complex in Marche region are present) and colonized the Adriatic islands as well as the mainland of the Dinaric karst. Subsequently, during the Pleistocene (2.5 Ma–11.7 ka), it presumably colonized again the Adriatic islands from the Dalmatian mainland. Its presence in the cave of Vodeni Rat was thus expected. However, the situation is more complex than it seems. As reported in Delić et al. (2020) and following an ongoing molecular revision, *N. hebereri* is composed of three distinct clades, which likely represent a complex of three cryptic species. The distribution of the three clades roughly follows the macro-geomorphological subdivision of the Adriatic coast of Croatia in North, Central, and South according to Bognar (1999). Our molecular analyses place the Vodeni Rat population clearly within the southern clade of *N. hebereri*.

Thermosbaenaceans

Thermosbaenaceans are considered Tethyan relicts widely distributed in different groundwater habitats across the world (Wagner, 1994). With 35 species known so far, their distribution covers the shallow Mediterranean Sea or its coasts. *Tethysbaena halophila* Karaman, 1953 (Figs. 3A and 9B) has been frequently found in wells and caves of the Croatian mainland and islands (Sket, 1986, 1988; Wagner, 1994). This species tolerates low oxygen conditions, the presence of H₂S, and a wide range of water salinity (from 0.5 to 30‰). Under polyhaline and mesohaline conditions (salinity from 18 to 30‰ and 5-18‰, respectively) the populations of *T. halophila* are quite dense whereas only a few individuals occur at lower salinity conditions (0.5-5‰) (Sket, 1986). *T. halophila* frequently occurs with copepods of the genus *Diacyclops*, and amphipods of the genera *Hadzia* and *Niphargus* (Wagner, 1994). In the Cave of Vodeni Rat, a total of 4 specimens were collected, all in the upper freshwater layer of the cave (Figs 3A and 9B). Nevertheless, despite no specimens were found in the DEEP and BOTTOM samples with salinity of 38.7-39.0‰ (Fig. 6), its presence in the brackish habitat cannot be ruled out since it was not sampled in this first survey of the Vodeni Rat Cave. Therefore, the presence of *T. halophila* in the cave of Vodeni Rat can be eventually verified by further sampling the transitional brackish water interval from about 5 to 9.5 m water depth and salinity ranging between 5 and 30‰ (Table 2).

DISCUSSION AND CONCLUSION

The peculiarity of the Vodeni Rat Cave is that until the 4th-7th century CE it was not an anchialine cave, but an ordinary dry karstic pit with freshwater of meteoric origin at the bottom, some 24 m below mean sea level. The cave was isolated from the surrounding phreatic environment by speleothemic calcite, which coats the inner walls of the cave, and fills fractures and joints crisscrossing the bedrock limestone. An U-Th date of 305 kyr of speleothemic calcite taken from one of these fractures in the immediate vicinity of the cave entrance, suggests that the Cave of Vodeni Rat has been sealed since the late Middle Pleistocene. This suggests that limnicoid stygobitic crustaceans such as the amphipods *Niphargus hebereri* and *Salentinella balcanica* and the thermosbaenacean *Tethysbaena halophila*, which are found today in both freshwater and anchialine habitats along the eastern Adriatic coast, entered Vodeni Rat Cave prior to 305 ka, when this epigenic cave was located well above sea level in this subsiding part of the Dinaric foreland. Similarly, two specimens of the copepod species *Diacyclops slovenicus*, which is known only from freshwater habitats in caves throughout the inner Dinaric karst, were found in the upper freshwater layer of the cave, suggesting that it colonized the cave during the late Middle Pleistocene, and in any case not after the Early Medieval Period. A similar origin can be inferred from the abundant presence of copepod *Halicyclops dalmatinus* in the freshwater layer of Vodeni Rat

Cave. Before this study, this species was documented in brackish water bodies in Montenegro and Apulia, suggesting that these copepods, as well as the ostracod *Pseudolimnocytere* sp., may have entered the Vodeni Rat cave in the late Middle Pleistocene.

A different story is the one told by the copepod *Speleophria mestrovi*. This species was first described in the Supurina Cave in Vis Island by Kršinić (2008) in a highly saline habitat (~37.0-38.0‰). In Vodeni Rat, *S. mestrovi* was found abundant in the most saline part of the water column (38.8‰) at about 14 m below mean sea level, and no specimens were caught in the freshwater part of the water column. This suggests that this species penetrated into the cave after the 4th-7th century CE when the cave became anchialine due to infiltration of highly saline waters probably from nearby tidally-influenced saturated cavities through newly open fractures and joints of the country rock limestone (Suárez-Morales et al., 2017).

The main question that remains to be answered is what happened between the mid 4th and the mid 7th centuries CE (i.e., the archeometric age span of amphora LR-1 found in the Vodeni Rat Cave) to the Momici and Soline Roman villas. The abandonment of these villas and the Vodeni Rat freshwater source may have something to do with the Late Antiquity Little Ice Age (LALIA 532-660 CE). This was a paroxysmal climatic crisis for humanity at a global scale in the troubled Early Medieval Period (i.e., the Dark Ages Cold Period or DACP, 4th to 10th century CE; Helama et al., 2017), which, in the middle of a long-term scenario of climate change, witnessed the fall of empires (e.g., the Roman Empire and the Eastern Turkic Empire; Fei et al., 2007; Buentgen et al., 2016), as well as migrations of entire populations from east to west and north to south in Europe and Asia (i.e., Vandals, Goths, Longobards, etc., with wars, conflicts, invasions, and destruction of preexisting social, political, and economical orders). More in particular, the LALIA period has been attributed to at least two exceptionally big volcanic events, which caused extreme yet different meteorological conditions in various parts of the world, such as droughts in mid and eastern Asia, torrential rains and floods in Japan, freezing cold in central Europe and northwestern Atlantic regions, cold and wet conditions in western Mediterranean regions, and "dry fogs" due to volcanogenic outgassing in various parts of the world (e.g., Stother & Rampino, 1983; Ludlow et al., 2013). The years-lasting, widespread dry fogs produced by the megavolcanic events of 532 and 626 CE would have set the conditions for acid rains, which may have had some effects on karstic regions by increasing carbonate dissolution on the surface as well as in fractures and joints like those surrounding the cave of Vodeni Rat until then sealed by cave calcite.

On the other hand, we should also consider the hypothesis by Evelpidou & Pierazzoli (2017, and references therein), that a very strong earthquake occurred in the northern Adriatic in 361 CE as part of an intense seismic sequence, which caused "... the coseismic subsidence of a known slightly submerged tidal notch that developed along over 200 km of the

Croatian shoreline". This tidal notch was also reported in the Jerolim Islet of the Pakleni Archipelago by Montanari et al. (2019) and may represent a taleteller of a strong earthquake capable of fracturing the limestone surrounding the Vodeni Rat Cave, thus terminating its long isolation from the sea and the birth of an anchialine environment. Practically, if the Vodeni Rat LR-1 amphora turns out to be an early model compatible with the 4th century CE, than the hypothesis of Vodeni Rat becoming an anchialine cave after "tectonic perturbations", as Mesić (2006) originally proposed, becomes plausible.

In conclusion, our study of the stygobitic crustacean fauna in the Vodeni Rat neo-anchialine cave revealed the presence of freshwater species that were present in this cave since at least the late Middle Pleistocene before they were isolated from the rest of the surrounding hypogean karstic environment in a sort of speleothem-sealed time capsule. These species may have had a history different than that of conspecific populations, which adapted to living in anchialine caves of the Croatian karst that got invaded by seeping seawater in much earlier times. Verification of this hypothesis will require future comparative genetic analysis of all the species found in the Vodeni Rat Cave and those known to inhabit anchialine caves along the Croatian coasts as well as caves in the inner Dinaric karst.

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