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Research Article

Faeces of *Rhinolophus euryale* (Chiroptera) from the winter season contain inorganic matter

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Abstract

Hibernating mammals arouse periodically from their torpor under the influence of an unknown mechanism to perform activities necessary for the correct functioning of metabolism. Our model species, the Mediterranean horseshoe bat (*Rhinolophus euryale* Blasius, 1853), wakes up during the winter and produces both typical consumptive as well as non-consumptive faeces (produced after no feeding activity). The aim of this study was to characterize the composition of the latter droppings of *R. euryale* in comparison to summer droppings to better understand the processes involved in such arousals from winter hibernation. The non-consumptive samples were morphologically similar and consisted of mucous material on the outside and a homogeneous mass inside. The internal homogenous mass inside the faeces was composed of organic as well as inorganic material, consisting of phosphate, calcium carbonate, quartz particles, and clay. We also confirmed the overall presence of faecal spherulites, calcite crystals and siliceous needles. The faeces contained no cells or if some, merely fragments of them. In contrast, summer faeces were composed of insect fragments; i.e. they were typically consumptive. In addition, we found no trace of insect-prey DNA in the winter droppings, which contained bat DNA instead. We also found peptides belonging to Mammalia as well as to other craniate and eukaryotes, but Arthropoda peptides occurred in only a rudimental occurrence. We found Bacteria peptides as well. Internal parasites were also visually retrieved. The high concentration of inorganic material and virtual lack of prey observed in the non-consumptive faeces indicate that drinking as well as direct sediment consumption occur inside the cave environment during the hibernation period. We conclude that winter arousals are unlikely to be aimed at gaining energy through foraging but most likely allow regulating water balance by active drinking.

Introduction

Bats in temperate regions enter torpor to reduce high-energy requirements during low-temperature and prey-shortage periods in winter (Geiser, 2004). However, the hibernating mammals studied to date arouse periodically at intervals ranging from several days to a few weeks during the winter season (Daan, 1972; Thomas et al., 1990; Lyman et al., 2013). Torpid individuals arouse spontaneously and increase body temperature dramatically, even if environmental conditions have not changed. A single period of torpor can last as long as 350 h, but periods of arousal rarely exceed 24 h (Geiser and Ruf, 1995). Arousals are energetically expensive, and bats use 75–90% of their stored fat for this recurrent activity (Thomas et al., 1990; Dunbar and Tomasi, 2006; Jonasson and Willis, 2012). Thus, arousals seem to be a necessary process involved in important functions, such as drinking, producing urine or faeces, sleeping, resisting diseases, foraging, mating or changing hibernation site (Avery, 1985; Brigham, 1987; Daan et al., 1991; Thomas and Cloutier, 1992; Thomas and Geiser, 1997). Physiological functions, such as the elimination of metabolic waste products, replenishing the blood glucose level or restoring cellular electrolyte balance, are achieved (Fisher, 1964; Galster and Morrison, 1970).

Winter arousals can lead to foraging behaviour or drinking and commuting flights between the hibernaculum and foraging areas (consumptive arousals). This occurs mainly when meteorological conditions improve. There is evidence that Rhinolophid as well as Vespertilionid bats feed on mild winter nights (Ransome, 1968, 1971; Hope et al., 2014; Williams et al., 2010; Zahn and Kriner, 2014). By selecting a roosting position that fluctuates with ambient temperatures and by maintaining arousal synchronization with dusk, *R. ferrumequinum* is able to take advantage of foraging opportunities when they occur.

On the other hand, bats often remain in their roosting cave while active (Park et al., 1999), indicating that foraging is unlikely to be a primary function of some arousals (non-consumptive arousals). The nature of these arousals is not clear. Miková et al. (2013) reported that the *R. euryale* produced two types of faeces during the winter: some containing prey remains (consumptive faeces) and some lacking prey remains — hereafter non-consumptive faeces. Records of similar bat faeces have been reported only twice in the literature (Whitaker and Rissler, 1992, 1993). However, the reason for defecating without apparently having eaten is not very well understood in bats.

In this study, we aim to give insight into the factors underlying the production of non-consumptive faeces and alongside to provide some clues for better understanding bat metabolism during torpor. To achieve these goals we performed a detailed analysis of the content of non-consumptive faeces produced by *R. euryale* during the hibernation period.

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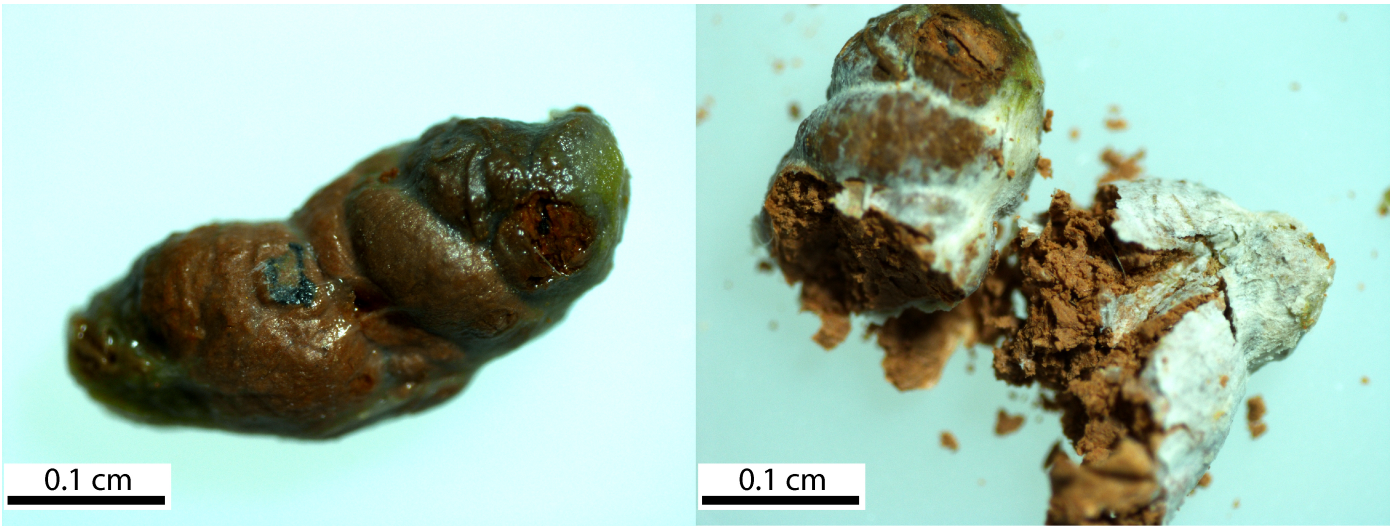

Figure 1 – A non-consumptive faecal pellet of *R. euryale* from winter in Baradla Cave, Hungary.

Materials and methods

Sample collection

Faecal samples were collected from a foil placed beneath a hibernating colony during one night (29 January 2014) in the biggest known hibernaculum of *R. euryale* in Central Europe, the Baradla Cave (NE Hungary, 340 m a.s.l., 48.5° N, 20.5° E; Uhrin et al., 2012). This colony is monospecific from a long-term point of view, and the number of hibernating *R. euryale* on the collection night was 4231. We randomly collected 180 faecal pellets and stored them in 98% alcohol. Typical summer faeces (30 pellets) were used for comparison and were gathered from *R. euryale* individuals captured during a mist-netting survey in the Jasovská jaskyňa Cave (SE Slovakia, 255 m.a.s.l., 48.7° N, 21.0° E) on 21 August 2012. The two locations are close to each other and both belong to the Gömör-Torna/Gemer-Turňa Karst area, which is the southernmost part of the inner limestone zone of the Western Carpathians.

Morphological analyses

Classification of faeces into non-consumptive and consumptive

The 180 winter faecal pellets and 30 summer pellets were first analyzed visually under a binocular magnifier. According to their general appearance, shape, structure, consistency and content, the pellets were classified into non-consumptive faeces and typical consumptive faeces (Miková et al., 2013).

Histological analysis

We analyzed histologically 66 non-consumptive winter faecal pellets and 10 typical consumptive pellets from summer. The faecal samples were dehydrated and routinely prepared for histological examination. Faecal sections (5 µm thick) were obtained with the aid of a rotary microtome and stained with haematoxylin-eosin (H/E, Martoja and Martoja-Pierson, 1970) for examination under a light microscope. Mucopolysaccharide (MPS) histochemistry was carried out (Alcian Blue –AB– at pH=2.5, Bancroft and Gamble, 2002) to localize carboxylated and sulphated MPS.

Petrographic analysis

We compared the composition and textural characterization of 66 non-consumptive faecal pellets and 10 typical consumptive faecal pellets from summer using an Olympus BH2 petrographic microscope equipped with an Olympus DP10 digital photo system. Microscopic slides were made from paraffin-thin sections. Calcite crystals were characterized through reaction with hydrochloric acid.

X-ray Diffraction

The whole mineralogy of the non-consumptive faeces was determined by X-ray diffraction (XRD), where 5 faecal samples were crushed and smoothed manually in agate mortar and one analysis was applied by a PANalytical X'pert PRO powder diffractometer equipped with a copper pipe (lCuKamedia=1.5418 Å, lCuKa1=1.540 60 Å and lCuKa2=1.544 39 Å), a vertical goniometer (Bragg-Brentano geometry), a programmable divergence slit, an automatic sample, a secondary graphite monochromator and a PIXcel detector. The measurement conditions were 40 kV and 40 mA with a weight between a 5 and 70° 2-theta sweep. We used a monocrystalline silicon "zero line" specimen. We processed the resulting diffractograms and identified phases using the PANalytical X'pert HighScore specific software in combination with the PDF-2 database of the ICDD. A semiquantitative estimate of the mineral content were conducted according to the "reflective powers" method of Schultz (1964) by means of measuring the areas of the diffraction peaks characteristic for each mineral.

Molecular analyses

DNA analysis

We carried out DNA analyses of 12 non-consumptive faecal pellets. DNA was extracted from each faecal pellet using the QIAamp DNA Stool Mini Kit (Qiagen Ltd., Crawley, West Sussex, UK) following the manufacturer's instructions with some modifications (Zeale et al., 2011). In order to detect the presence of bat DNA, a partial sequence of bats' mitochondrial 16S rRNA gene was amplified from faecal DNA extracts using the primers employed by Van Den Bussche and Hoofer (2000) and successfully used in a variety of bat species (e.g. Gu et al., 2008). We performed a second PCR to detect insect DNA in the faecal samples using the insect generic COI primers ZBJ-ArtcF1c and ZBJ-ArtR2c (Zeale et al., 2011), which yield an amplicon of ca. 157 bp located within the 5' end of the standard 658 bp COI barcode region (cytochrome C oxydase subunit I of mitochondrial DNA). Positive and negative DNA controls were included in both PCR approaches. Information regarding PCR conditions and sample visualization are detailed in the supplementary material (S1).

Protein analysis

Processing and analysis was done in parallel for both non-consumptive and consumptive faecal samples. Ten pellets (total weight 50 mg) of the non-consumptive faeces and 10 pellets (42 mg) of the consumptive summer faeces were analyzed. The amount of protein was quantified, and the same amount of protein was digested and analyzed for each sample by liquid chromatography-tandem mass spectrometry (LC-MS/MS, Aebersold and Mann, 2003). Information regarding protein analysis procedures and conditions are specified in the supplementary material (S1). Finally, the UniProt Eukaryota, Mammalia and Arthropoda (version 2014_10) reference databases were used to compare the proteins observed from the faeces. Proteomes of non-model organisms are usually poorly represented in protein databases, and only 23

proteins are reported for *R. euryale* in UniProtKB (www.uniprot.org). Therefore, larger databases including different taxonomical groups were used to allow for cross-species peptide and protein identification. Mammalia and Arthropoda protein databases were used to differentiate peptides belonging to *R. euryale* and their prey.

Results

All collected winter pellets (N=180) were composed of the mucous material on the outside and the blackish-brown homogenous matter inside (Fig. 1); they were morphologically similar to each other and their appearance matched the description given by Miková et al. (2013) for winter non-consumptive faeces. All summer samples (N=30) were typical consumptive faeces and were composed of insect fragments (mainly Lepidoptera).

We found no preserved tissue structures or cells in the non-consumptive winter faeces and only small cellular fragments were observed. Specific histochemical staining of the non-consumptive faeces highlighted the presence of mucous material on the outer side, a feature that was missing in the consumptive summer faeces. Regarding the consumptive summer faecal pellets, they were composed of insect fragments; they formed a large porous texture without signs of any inorganic matter or mucus (Fig. 3A). On the other hand, the non-consumptive winter faecal pellets showed a compressed structure made up of a homogeneous silty mud groundmass of phosphates, organic matter, quartz particles and clay surrounded by a mucous layer (Fig. 3B, C), in which the groundmass was composed of around 30% organic and 70% inorganic matter. Furthermore, the mineral part was composed of 43% clay, 33% calcite and 24% quartz. Microscopic spherulites of about 5 µm to 8 µm in diameter, present in all analyzed non-consumptive pellets and showing high birefringence and permanent cross extinction in crossed polarized light (Fig. 3D), were identified as calcium carbonate faecal spherulites. We also found siliceous needles 0.05 mm to 0.2 mm in length (Fig. 3E, F) identified as sponge, octocoral or echinoderm specula in all analyzed non-consumptive pellets. We confirmed the presence of 0.25 mm to 0.55 mm columnar calcite crystals in 10 of the 180 winter faeces (Fig. 3H). Finally, we found a negligible amount of arthropod remains in the analyzed pellets (Fig. 3G) that we were not able to classify to any lower taxa. In the morphological analysis we also found an individual of Nematoda and hairs.

Regarding molecular analyses, we found bat DNA in 10 out of the 12 non-consumptive faecal samples but no insect DNA was detected in them. We identified a total of 382 and 297 eukaryotic peptides in the non-consumptive and consumptive faeces. In the non-consumptive faeces there were 313 mammalian peptides and only 12 arthropod peptides. Conversely, in the consumptive faeces the mammalian peptides decreased to 129, while arthropod peptides soared to 134 (Fig. 2). The ratio of arthropod to mammalian peptides changed from 0.04 to 1.04, a 26-fold increase between non-consumptive and consumptive faeces, respectively.

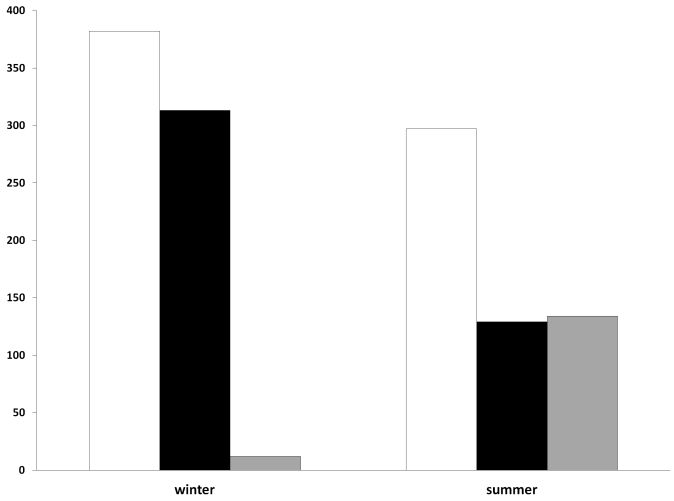

Figure 2 – Number of peptides identified in *R. euryale* faecal samples from winter and summer. White – eukaryotic peptides, black – mammalian peptides, grey – Arthropoda peptides.

Discussion

Our comprehensive multidisciplinary analysis of winter non-consumptive bat faeces of *R. euryale* has shown that most of them consisted of inorganic material, and thus they were unlikely to be the result of prey digestion. To our knowledge, only Whitaker and Rissler (1992, 1993) have described a similar type of winter guano. They recognized faecal plugs, particulate material and white material within the gastrointestinal tract of bats during winter. They concluded that the brown sandy material was of insect origin, the result of chitin digestion. However, our results clarify that the brown particulate material is not of insect origin. In fact, histological analysis has shown that there were almost no arthropod remains in these non-consumptive winter faeces; just a few were found and are likely to be indigestible chitinous debris from earlier foraging bouts. Most of the material in these faeces consists of inorganic matter surrounded by mucous material absent in consumptive faeces. This inorganic matter found in non-consumptive faeces was composed of clay, calcite and quartz, similar to the composition of the cave sediment itself (Berényi Üveges et al., 2006). Spherulites, like those found in all winter bat droppings, are common in ruminant herbivores (Canti, 1997, 1998, 1999), which produce spherulites from the calcium they intake along with water or food. We herein confirm their presence in bats. These faeces also contained siliceous needles identified as sponge, echinoderms or octocorals specula (Flügel, 2004), as well as formed columnar calcite crystals typical for speleothem formations in lower occurrence (Frisia and Borsato, 2010; Fairchild and Baker, 2012). Cave waters are likely the source of faecal spherulites, since cave waters have a high content of calcium bicarbonate (Borbás et al., 2011). Similarly, the siliceous needles and the columnar calcite crystals found in the bat faeces may also have their origin in cave waters or sediment. Specula from sponge or echinoderms are present in the Triassic limestone of the Aggtelek Karst (Senowbari-Daryan et al., 2011), and they can appear in the cave as insoluble parts of the limestone, while columnar calcite crystals could have been formed in the Baradla Cave speleothems (Lauritzen and Leél-Ossy, 1999; Szeidovitz et al., 2008). Although we found some chitinous debris, we did not detect any arthropod DNA in any of the analyzed non-consumptive faeces. Several molecular diet studies have shown that arthropod prey DNA can be clearly detected from bat faeces (Zeale et al., 2011; Clare et al., 2011; Razgour et al., 2011; Alberdi et al., 2012) including those of *R. euryale* (Arrizabalaga-Escudero et al., 2015) or even from half a bat pellet (Bohmann et al., 2011). In contrast to Whitaker and Rissler (1992, 1993), the chitinous traces found in our samples were probably old indigestible hard cuticles with no attached DNA to be extracted. In accordance, arthropod peptides were almost absent in the non-consumptive winter faeces, in sharp contrast with their significant number in the summer faeces. Furthermore, we did not find any kind of preserved cells in the non-consumptive faeces. However, we successfully amplified bat DNA from these faeces. Although PCR amplicons were not sequenced, considering the large size and the monospecific nature of the hibernation colony (Uhrin et al., 2012), faeces were very likely produced by *R. euryale*. This DNA probably came from traces of gut epithelial cells present in the mucus.

The production of non-consumptive faeces in *R. euryale* might be linked to regulation of water balance by active drinking (or most probably licking). This is consistent with some studies stating that evaporation and water loss seem to be the main causality for arousals (Thomas and Geiser, 1997; Neuweiler, 2000; Ben-Hamo et al., 2013). Park et al. (2000) suggested that bats remain torpid until a threshold level of metabolic (or possibly water) imbalance occurs. Even if intermittent feeding activity through the hibernation period permits bats to obtain water (Speakman and Racey, 1989), bats drink opportunistically without moving far from their hibernacula, and some species have been ob-

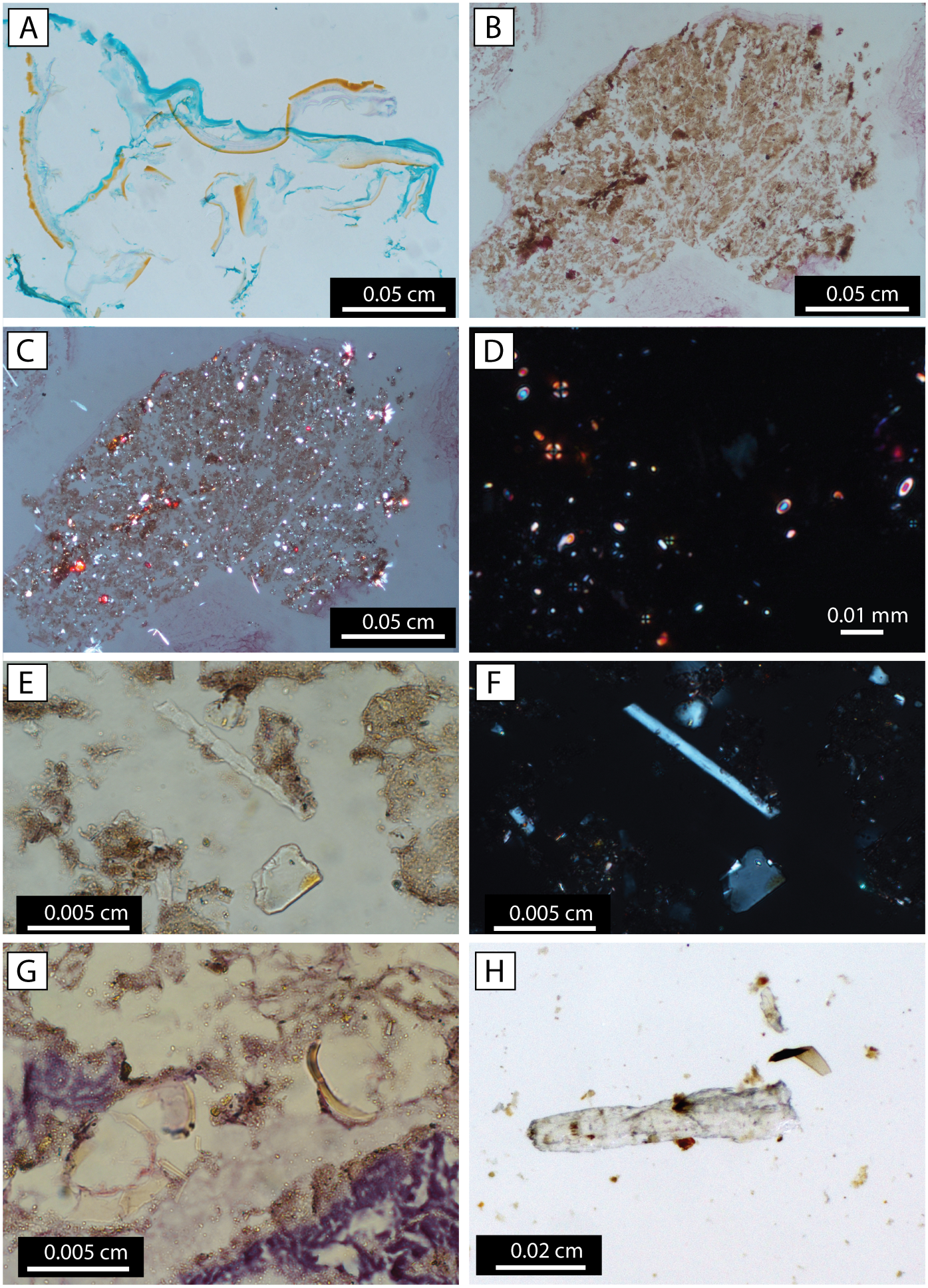

Figure 3 – Micrograph of *R. euryale* faeces from Baradla Cave: A) General summer pellet with chitinous remains; B) General winter pellet; C) Same visual field as (B) under crossed polarised light; D) Calcium carbonate spherulites in winter faeces, under crossed polarised light; E) Siliceous needle and some quartz grain in winter faeces; F) Same visual field as (E) under crossed polarised light; G) A few parts of unidentified insects in winter faecal samples; H) A columnar calcite crystal found in a winter pellet.

served drinking the standing water available in their caves (Twente, 1955; Speakman and Racey, 1989; Boyles et al., 2006). Most bats hibernate in humid caves or mines, where they have access to drinking water, and they have been observed drinking or licking condensed water when aroused (Davis, 1970). In light of this, intentional geophagy cannot be ruled out. Tropical frugivorous bats have been observed visiting natural mineral licks (Tuttle, 1974; Voigt et al., 2007, 2008; Bravo et al., 2008, 2010). In south-eastern Peru, these bats drink on the wing from puddles or pools that form in depressions left by terrestrial vertebrates, and Bravo et al. (2010) hypothesized that bats intentionally lick soil alongside water.

Moreover, inorganic matter not only serves as a supplementary source of minerals (Davies and Baillie, 1988; Klaus and Schmid, 1998) but also protects against toxins (Diamond, 1999; Gilardi et al., 1999; Brightsmith et al., 2008), against indigestion and protects cells by adsorbing intestinal insults and enterotoxins (Mahaney et al., 1997; Dominy et al., 2004). Additionally, it adds a mechanical advantage in the digestion process (Brightsmith and Munoz-Najar, 2004). Therefore, frugivorous species may lick soil or water as reliable secondary sources of limited nutrients (Brightsmith et al., 2008; Emmons and Stark, 1979). Calcium is a limiting element for correct physiological functioning in small mammals. Thus, Bravo et al. (2008) suggested that bats visiting collpas in Peru seek to refill calcium levels in addition to obtaining water. This might be related to the need to expel the wastes produced by metabolizing fat stores during torpor or might be involved in the discarding of dead cells from the gastrointestinal tract. Because we found a remarkable amount of mucous material surrounding the pellets, it is plausible that bats may ingest inorganic matter in order to keep the gastrointestinal tract functional or to prevent it from collapsing and atrophying. This may help them take advantage of potential foraging opportunities that appear during the winter.

Rhinolophus euryale is able to hunt on milder winter nights, but during winter it produces two types of faeces: with and without prey remains (Miková et al., 2013). The high concentration of inorganic material and virtual lack of prey observed in the non-consumptive faeces indicate that drinking and also direct sediment consumption occur inside the cave environment. Bats can take sediment from their claws when grooming, collect it directly from their foot (Kolb, 1982) and lick it from different surfaces. This behaviour does not seem to be displayed during foraging bouts and is probably connected with water metabolism.

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Supplemental material S1 DNA analysis and protein analysis protocols.