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SHORT NOTE

Crested porcupines (*Hystrix cristata*): mycophagist spore dispersers of the ectomycorrhizal truffle *Tuber aestivum*

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Abstract

Truffles, as hypogeous, ectomycorrhizal fungi, have no means to actively discharge spores into the environment and thus depend on mycophagists for spore dispersal. After consumption of fruiting bodies by animals and passage through the digestive tract, the spores are released in faecal pellets. Recently, in the Abruzzo region (Italy), *Hystrix cristata* has been spotted inside private truffières, but its role in spore dispersal has never been investigated. Here, we report our research on the occurrence of *Tuber aestivum* spores in porcupine's faecal contents in a truffière in L'Aquila, Italy, where a *H. cristata* specimen was photographed. The spores were isolated from faeces by using a suspension of 0.7 M $ZnSO_4$. We also verified degradation and disfiguration of the digested spores' reticular ornamentation compared to that of fresh spores from ascomata collected inside the truffière, through measurements performed by scanning electron microscopy. A few truffle spores had germinated within the faeces.

Keywords Mycophagy · Truffles · Faeces · Spores · Spore germination

Introduction

Sequestrate fungi are mostly fleshy, hypogeous, ectomycorrhizal species descended from epigeous mushroom ancestors as fruiting bodies (truffles) that produce their spores sequestered within the surrounding tissues (Trappe et al. 2009). Truffles cannot actively discharge their spores into the surrounding environment and thus mainly depend on mycophagous animals for spore dispersal.

The transition from epigeous to hypogeous fruiting habit favoured the success of this dispersal mode (Bonito et al. 2013). The production of a strong aroma to attract animals represents one of the mechanisms evolved as adaptation by truffle species (Pacioni et al. 1991). Truffles of the genus *Tuber* (Pezizales, Ascomycota) may also contain endocannabinoids and their precursors, such as phosphatidylethanolamine (PE) (Mannina et al. 2004) and anandamide (*N*-arachidonoylethanolamine, AEA) (Pacioni et al. 2015), that entice animals to truffle consumption.

Tuber spp. are ectomycorrhizal symbionts with roots of gymnosperms and angiosperms and are the most commercially valuable hypogeous fungi. *Tuber aestivum* Vittad. is among the most cherished truffles worldwide (Bonito et al. 2010) and is the most widespread European *Tuber* species (Gryndler et al. 2011; Hall et al. 2007). Its ecological amplitude (Stobbe et al. 2013) and distribution from North Africa in the south to Gotland (Sweden), in the north and Spain in the west to Turkey and the Caucasus in the east (Song et al. 2005) make it exceptionally suitable for cultivation. Its population sustainability in the long term depends on the sustainability of spore dispersal by mycophagous animals.

Mycophagists include a huge variety of invertebrates and vertebrates that rely on fungi to a greater or lesser extent for food, with more than 250 species of vertebrates alone as recorded in the literature (Elliott and Trappe, unpublished data). Mycophagists were classified by Claridge and Trappe (2005) according to their reliance on fungi for food as obligate, preferential, casual or accidental. Mycophagy can be studied by analysis of stomach contents or examination of faecal pellets (Maser et al. 1978; Cazares et al. 1999). Through microscopic

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analysis of the ingested material, certain modifications in the spore morphology can be discerned. The degree of spore degradation depends on the species of mycophagous animal and their diverse features, e.g. body temperature and gut retention (Colgan and Claridge 2002).

Hystrix cristata L. (crested porcupine) is a big rodent of the *Hystricidae* family, which includes 20 species of Old World porcupines (Walker 1964). This rodent belongs to subgenus *Hystrix* together with two other species: *H. indica* and *H. africaeaustralis*. It is distributed in northern and sub-Saharian Africa (Mohamed 2011). Outside the African continent, Italy is the only country hosting this species. There, it has been listed among protected species since 1977 (National Law 968/1977). The crested porcupine was introduced to Italy from North Africa by Romans 2500 to 1500 years ago (Trucchi and Sbordoni 2009). During recent decades, its range has seen a major expansion to northeastern Italy. The northern limit is the province of Verona (Veneto region) to the east and to the west by the province of Piacenza (Emilia Romagna region) (Amori et al. 2008). In Abruzzo, *H. cristata* is present inland into L'Aquila and Teramo provinces (Pellegrini et al. 1992; Febbo and Pellegrini 1994; Morini 2011) and the Majella National Park (Santoleri and Cerceo 2007).

Crested porcupines are mainly herbivorous, mostly eating underground storage vegetal organs (i.e. bulbs, tubers, roots), fallen fruits and cultivated crops (Santini 1980). Their nourishment appears to be primarily related to seasonal availability (Bruno and Riccardi 1995; Mohamed 2011). It has not been included in the list of mycophagous animals, but sightings and reports inside truffières in the province of L'Aquila are increasingly frequent. We hypothesised *H. cristata* to be a potential truffle consumer and tested this hypothesis by analysing faeces belonging to this species collected in a *Tuber aestivum* truffière.

Materials and methods

Study area and faecal sampling

In 2017, a porcupine was photographed (Fig 1S) in a fenced *T. aestivum* truffière in Tempera (L'Aquila) (42° 22′ 32.5″ N, 13° 26′ 29.9″ E), so the area was selected for this research. This plantation is 30 years old, covers an area of 0.25 ha and contains 50 *Quercus pubescens* Willd. and 50 *Corylus avellana* L. plants. Faeces of *H. cristata* were collected from May 10 to August 15, 2017, for six surveys, with two to six samples collected each time.

Preparation of faecal samples for spore detection and microscopic analysis

Samples were collected and placed directly into separate sterile plastic bags, classified by date and stored in a freezer. In the laboratory, faeces were diluted with sterile distilled water and the suspension was decanted for 1 hour. The precipitate was then poured through sieves with mesh diameter 400 and 150 μm. The material under 150 μm broad was centrifuged (1500 rpm, 3 min). The supernatant was drained and 40 ml of a 0.7 M $ZnSO_4$ solution was added to the pellet and spun for a few seconds. Additional $ZnSO_4$ solution was added until the liquid surface reached the tube rim level. Floating truffle spores were then recovered by placing a microscope slide on the tube for 15 min. The slides were then covered with a cover slip and examined under a microscope (Zeiss, Axiostar plus, Oberkochen, Germany) at × 400. Each sample was examined in triplicate.

After a first microscopic analysis, fresh spores of *T. aestivum* collected in the same truffle ground and spores digested by the porcupine were visualised by scanning electronic microscopy (SEM) to assess the morphology of spore surfaces and ornamentation. A drop of spore suspension in water was placed on an aluminium SEM stub (diameter 12 mm). After drying, the samples were sputter-coated with a thin gold film using an AGAR automatic sputter coater (20 mA, 45 s) and examined by use of a SEM Philips XL30 CP. Forty measurements of spore ornamentation thickness were taken for both spores from fresh fruiting bodies collected inside the truffière and spores from the porcupine faeces.

Statistical analysis

The size of spore ornamentation was analysed by one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). Analyses were conducted with XL-STAT software (Addinsoft Inc., New York, NY, USA).

Results

The *T. aestivum* production of the truffière under investigation took place from May to July 2017, for a total production of 20 kg distributed in about 800 fruiting bodies. Ascomata production was regularly distributed inside the truffière, and the porcupine faeces were collected in the entire area.

The collected faeces contained mostly plant material. Among this material, 302 *T. aestivum* spores were found. No truffle spores were detected until June 20. Spores were found freed from the asci in 94.3% of the total, whereas 5.7% were still contained within the asci. Four spores from within the faeces had emergent germ tubes (Fig. 1a, b). Compared to the well-defined reticulum of a fresh spore (Fig. 1c), the SEM micrograph of a partially digested spore reveals notable degradation and disfiguration of its reticulum ridges, which averaged less than half the thickness of that of the fresh spores (Figs. 1d and 2).

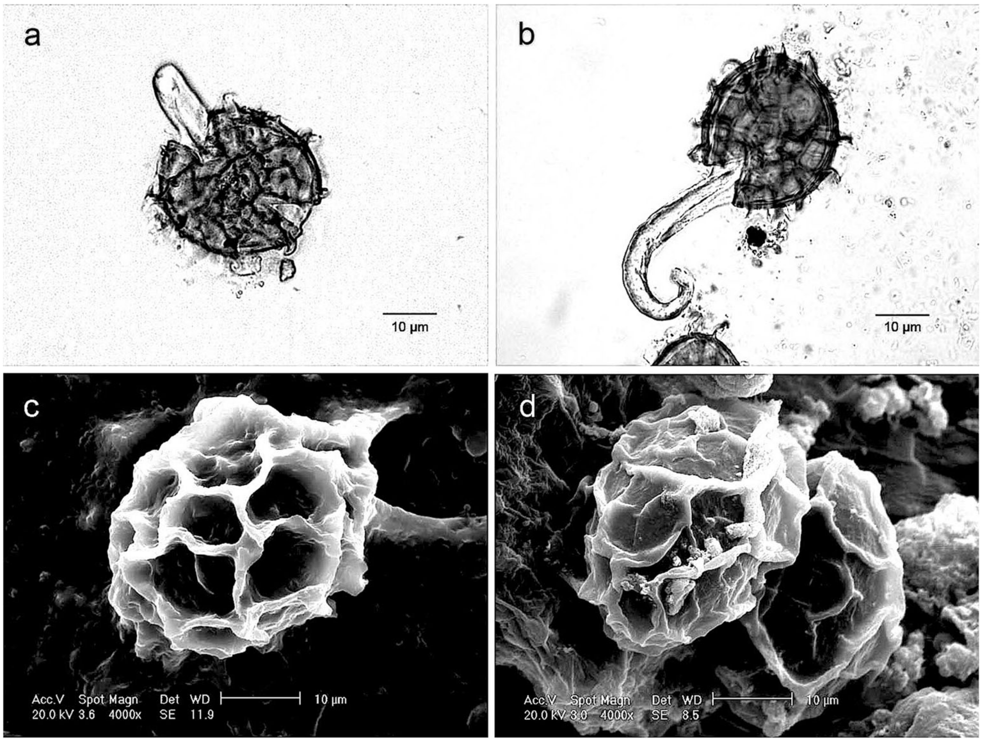

Fig. 1 *Tuber aestivum* spores. **a**, **b** Light micrographs, germinated, isolated from *Hystrix cristata* faeces. **c**, **d** Scanning electron micrographs of spores **c** from fresh ascoma and **d** from faeces, showing degeneration of reticular ornamentation due to digestion by *Hystrix cristata*

Discussion

This study reports the first evidence of *H. cristata* mycophagy. Mycophagy by small mammals has been studied worldwide: most small mammal species studied so far have been rated as obligate or preferential mycophagists (Claridge and Trappe 2005; Maser et al. 2008; Urban 2016). Among big mammals, Ashkannejhad and Horton (2006) and Nuñez et al. (2013) reported the consumption of sequestrate fungi as *Rhizopogon* spp. as well as epigeous *Suillus* by wild boars and deer while Piattoni et al. (2012) demonstrated *Tuber* consumption by wild boars. Mycophagy of *Tuber* spp. has also been reported for murids, the most species-rich family of rodents, sciurids and glirids (Schickmann et al. 2012; Currah et al. 2000; Urban 2016). This phenomenon has even been observed in Australia, where the genus *Tuber* is not native but has been introduced along with exotic pines: spores of *Tuber californicum* Harkn. appeared in faeces of several Australian native bush rats, *Rattus fuscipes*, sampled in a *Pinus radiata* plantation that pine being native to California (Trappe, unpublished data).

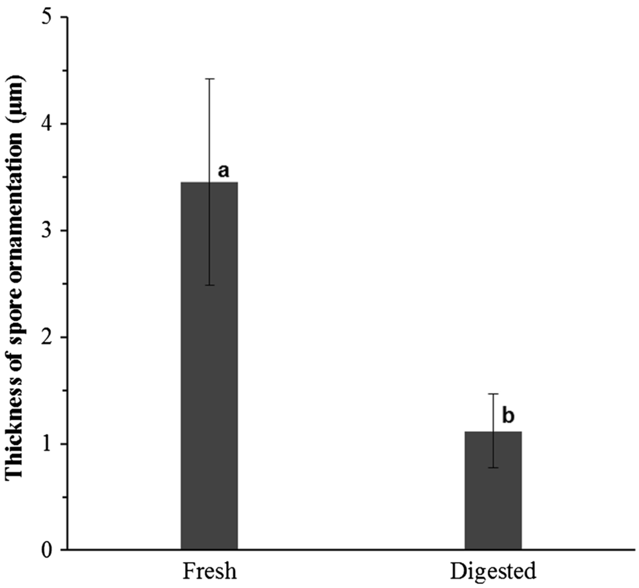

Fig. 2 Average thickness of walls of fresh and digested spore reticular ornamentation of *T. aestivum*. Vertical bars represent the standard deviation

The results of our survey confirm that *H. cristata* consumes truffles. Although the sampling periods occurred during summer, when food that typically constitutes *H. cristata* diet abounds, our data show that the porcupine definitely seeks truffles.

In our truffière, a ditch under the fence appeared for the first time in May 2017 and very likely the porcupine itself dug this ditch to feed on *T. aestivum*.

Truffle aromas can diffuse for considerable distances under good weather conditions and *Tuber aestivum* is among the *Tuber* spp. noted for production of strong aromas in concert with its associated microorganisms (Vahdatzadeh et al. 2013). Our results show that from 10 May 2017 to 15 June 2017, when truffles would be mostly immature, no spores appeared in the faeces. Instead, the following sampling period (20 June 2017–15 August 2017) was when all the spores were found.

Clearly, the porcupine fed on truffles when they were reaching maturity. At that time, most to all asci contain completely developed spores and fruiting bodies produce the strong aromas that attract mycophagists (Splivallo and Culleré 2016).

In the July–August samplings, the four germinated *Tuber* spores detected in *H. cristata*'s faeces are, so far as we know, the first report of germinated *Tuber* spores in animals' faeces.

As demonstrated by Piattoni et al. (2014), the passage of truffles through a pig's digestive tract not only maintained the spores' vitality but also increased their effectiveness as ectomycorrhizal inoculum. Similarly, Trappe and Maser (1976) showed that *Glomus macrocarpum* Tul. & C. Tul. spores germinated a few days after extraction from faeces of a California red-backed vole, *Myodes californicus* (Merriam, 1890). Colgan and Claridge (2002) demonstrated that spores of *Rhizopogon* spp. (a hypogeous basidiomycete) in rodent faeces were effective ectomycorrhizal inoculum on *Pseudotsuga menziesii* (Mirb.) Franco and reviewed the substantial literature on effectiveness of faeces of diverse animals containing spores of a variety of hypogeous fungi as inoculum for diverse host species.

No germinated spores were reported in the faeces in the studies noted above. This suggests a hypothesis that the digestive apparatus of *H. cristata* enhances rapid germination of *Tuber* spores by possible degradation of spore walls that retards breaking of dormancy. The literature offers little information on the digestive system of *H. cristata*. However, the morphology of the digestive tract of *Hystrix africaeaustralis*, a South African species, has been studied by van Jaarsveld and Knight-Eloff (1984). The stomach is relatively large for a herbivorous rodent. The intestine forms a long and thin narrowing of the digestive tract, so that the daily amount of food ingested by the animal is reduced but the absorption and enzymatic activities are extended. The caecum and the colon together form a unique fermentation chamber, capable of digesting a small amount of fibre. We assume that the resulting prolonged enzymatic activity might have degraded spore ornamentation and wall to hasten breaking of spore dormancy. Analyses of digestibility of an array of fungi by Wallis et al. (2012) showed variation between species and discussed the variation between animal species in digestion physiology. Hindgut fermentation extends the time in the caecum for microbial hydrolysis of fungal cell wall material as described above for *H. africaeaustralis*. This microbial enhancement in digestion of fungal cells not only releases more nutrients to the animal but also promotes digestion of fungal cell walls, including spore walls. This aspect regarding the effect of porcupine digestion on degradation of spores needs further investigation.

H. cristata does not hibernate, consequently during winter, outside the growing seasons of its primary foods; it must switch to autumn and winter crops such as truffles, e.g. the many *Tuber* species and other hypogeous fungi: hypogeous fungi are notably high in nutrients (Claridge and Trappe 2005; Wallis et al. 2012).

Further studies are needed to demonstrate the mycophagous habits of crested porcupines towards other truffle species and the effect of porcupine digestion on infectivity of truffle spores for rapid colonisation of ectomycorrhizal hosts.

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