Journal of Insect Science (*2016*) 16(1): 94; 1–8
doi: 10.1093/jisesa/iew070
Research article

OXFORD

Feeding Patterns of *Tyrophagus putrescentiae* (Sarcoptiformes: Acaridae) Indicate That Mycophagy Is Not a Single and Homogeneous Category of Nutritional Biology

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Subject Editor: Sara Goodacre

Received 6 May 2016; Accepted 8 July 2016

Abstract

Mycophagy should not be considered as a single and homogeneous category of nutritional biology due to the specific symbiotic chitinolytic bacteria associated with mites and fungi. To test interaction among mites, fungi, and chitinolytic bacteria, experiments were conducted on the model species *Tyrophagus putrescentiae* (Schrank). *Mucor* sp*, Alternaria alternata, Penicillium claviforme, P. griseofulvum*, and *Verticillium* sp. were plated onto malt agar and offered to *T. putrescentiae* in the laboratory. Mites were evaluated utilizing microanatomical examination based on histology, excrement analysis using fluorescence microscopy, bacterial plating, impact of mite homogenate on fungi in Petri dishes, reproduction of mites feeding upon each fungus, and isolation of associated bacteria inside mites. There were clear differences regarding the digested spores of different fungi passing through the gut and subsequently in the feces. Abundances of bacterial cells in excrement also corresponded to the fungi offered. The extracts from mites had chitinolytic activity, and the plated bacteria are known to produce exochitinases. The various feeding patterns observed were caused by differences in the cell wall structures of the tested fungi. The study illustrates that mycophagy in saprophagous mites does not consist of a single pattern, but rather that it can be classified into several sub-patterns depending upon the digested fungal species and its parts. The results point to a nearly symbiotic relationship between chitinolytic bacteria and digested fungi in mycophagous microarthropods.

Key words: digestion, feeding habit, fungal food offer, specificity of chitinolytic bacteria, *Tyrophagus putrescentiae*

The food selection of saprophagous soil mites has been suggested as key for understanding the high diversity of soil microarthropods. Partitioning of fungal food resources may contribute considerably to soil animal species diversity (Anderson 1975). Diversity can therefore be evolved according to the food offer, as is characteristically the basis for diversification in the soil world. However, the lack of coevolution between saprophagous animals and soil fungi is contradictory to the high species diversity (Maraun et al. 2003). In addition, the partitioning of fungal food resources is of very limited importance (Maraun et al. 2003). This contradiction points to a need for methodical approaches to study food selection and preferences.

The first indication of some selection had emerged as a result of clearing mites in lactic acid for taxonomy purposes and analyzing gut contents. That process, however, revealed only corpuscular food in the gastrointestinal tract (fungi, plant fragments; Wallwork, 1976). Some morphological studies, such as those by Schuster (1956) and Kaneko (1988), had considered food preferences according to cheliceral structure. Studies by Czajkowska (1970) and Pankiewicz-Nowicka et al. (1984) were of an experimental nature and based upon cafeteria tests. The length of stay on the offered fungi and production of excrement were important in such studies. The fungi offered by those authors had been plated on malt agar substrates. Koukol et al. (2009) later offered forest fungi cultivated on a natural substrate of coniferous needles. While the consumption and subsequent excrement production were considered in those methods, the extent of digestion and palatability of fungi were neglected. Those authors had assumed the digestion and utilization of food to be an automatic process occurring after consumption and intake of food followed by the production of excrement. Some propagules, however, can be passed intact through the gut with no digestion occurring (Smrž and Čatská 1987).

The presence and detection of digestive enzymes with the ability to hydrolyze the main compounds in ingested food provide alternative tools to study food selection. Luxton (1972) tested three enzymes important for the decomposition of soil substrates: trehalase,

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chitinases, and cellulases. He established three food groups: *microphytophagous*—consuming microorganisms (fungi, bacteria, and algae), *macrophytophagous*—consuming plant litter, and *panphytophagous*—consuming both food types with no visible selection. His studies constituted the pilot work conducted on these topics. Luxton was followed by Zinkler (1972) and Haq (1981). A similar but more sophisticated study was conducted by Siepel and de Ruiter-Dijkman (1993), who established the following food *guilds* among saprophagous mites: (i) *herbivorous grazers*—with cellulase activity, digesting plant cell contents and walls, as well as algal cells; (ii) *herbivorous browsers*—without cellulase or chitinase activity, digesting also animal tissue, including carrion, and bacteria; (iii) *fungivorous grazers*—with trehalase and chitinase activity, digesting both cell contents and walls of fungi; (iv) *fungivorous browsers*—with trehalase activity only, digesting only cell contents of fungi, but also lichens with high content of algal tissues; (v) *herbo-fungivorous grazers*—with chitinase as well as cellulase activity, digesting fungal and plant cell contents and walls, as well as lichens; (vi) *opportunistic herbo-fungivores*—with trehalase and cellulase activity, digesting the cell walls of plants, fungal cell contents, and lichens; and (vii) *omnivorous mites*—with cellulase as well as chitinase activity, digesting fungal as well as plant cell walls, but not digesting trehalose.

The most recent methods were based on analyzing contents of labeled isotopes (Schneider et al. 2004a,b) or fatty acids characteristic of various food types (Ruess et al. 2002). The first of these approaches resulted in the following categories of feeding preferences: (i) carnivores, scavengers, and omnivores consuming living or dead animals and fungi; (ii) secondary decomposers consuming fungi and, in part, litter; (iii) primary decomposers consuming litter with poor fungal and bacterial colonization (i.e., nearly fresh litter); and (iv) phycophages and fungivores consuming lichens and algae. While both methods are very useful and accurate, they are not very particularized (Maraun et al. 2011).

Based on the aforementioned concept, a part of saprophagous mites (i.e., microphytophagous, panphytophagous, fungivorous or herbo-fungivorous grazers or browsers and omnivorous mites) include mycophagous species that are indeed able to consume and digest fungi, but there exists more detailed specialization in this respect (Schneider and Maraun 2005, Schneider et al. 2005). Mycophagous mites consume fungi in various ways, by (i) piercing only the cell wall and extracting the cell contents, for which chitinase activity is not necessary; (ii) consuming an entire cell, cutting it, but only extracting or drinking its contents, for which chitinolytic activity is not necessary; and (iii) consuming and digesting the whole cell, for which chitinolytic activity is necessary. A mite operating in the last manner can be classified as a *true mycophagous animal* (Smrž and Čatská 2010).

The various fungi differ particularly in terms of the chemical structures of their cell walls and more especially regarding the chitin in the cell walls. The diversity of chitin was confirmed and emphasized by Jollès and Muzzarelli (1999). There are many structural types of chitin and therefore many types of chitinolytic enzymes that are specific for such substrates. It was unclear for many years whether the production of chitinolytic enzymes was autochthonous or allochthonous (Luxton 1972, Siepel and de Ruiter-Dijkman 1993, Erban and Hubert 2008). The essential role of chitinolytic enzymes seems nevertheless to be clear, and no animal is able to digest chitin or similar substances (e.g., chitosan) without the availability of one or more chitinolytic enzymes (Jollès and Muzzarelli 1999). Of course, the molting of arthropods is accompanied by chitinolytic activity, even though the digestive function may be lacking (Merzendorfer and Zimoch 2003). The producers of chitinolytic enzymes are bacteria (several species), including actinomycetes (*Streptomyces* spp.), as well as some fungi (*Trichoderma* spp.) (Jollès and Muzzarelli 1999, Brurberg et al. 2001, Citterio et al. 2001, Kobayashi et al. 2002, Chang et al. 2003, Aktuganov et al. 2004, Kishore et al. 2005). Several chitinase-producing bacteria have been found as associated organisms inside saprophagous soil mites (Smrž and Trelová 1995, Smrž 2003, Smrž and Soukalová 2008, Smrž and Čatská 2010).

The presence of chitinolytic bacteria associated with mites suggest that mycophagy is not a single and homogeneous category of nutritional biology and that food selection exists in saprophagous soil mites consuming fungi due to the specific symbiotic chitinolytic bacteria associated with the mites and fungi. To test this hypothesis, the model true mycophagous species *Tyrophagus putrescentiae* (Smrž 2003) was selected for laboratory feeding experiments. *T. putrescentiae* inhabits field soil environments (Smrž and Jungová 1989) but is also a synanthropic mite in human habitations and stored food products (Czajkowska 1970, Pankiewicz-Novicka et al. 1984). The experiments were conducted by offering different fungi to *T. putrescentiae*. The parameters of food digestibility and palatability have been defined on the basis of histological, microanatomical, and microbiological facts (Smrž 2002a, Smrž and Čatská 2010) in combination with plating of bacteria isolated from the mites. All approaches were applied to compare the interaction among fungi, *T. putrescentiae*, and bacteria.

Materials and Methods

The following fungi from the reference collection were offered as food for mites: *Mucor* sp., *Alternaria alternata, Penicillium claviforme, P. griseofulvum*, and *Verticillium* sp. Fungi were plated onto malt agar (Fluka, Sigma-Aldrich, St. Louis, MO) in laboratory conditions (22°C, 70% relative humidity in the room).

Tyrophagus putrescentiae mites originated from laboratory culture maintained at the Crop Research Institute, Prague, Czech Republic. The mites were cultivated in IWAKI tissue culture flasks (Sterilin, Newport, United Kingdom) with filter cap plugs on diets consisting of wheat germ, oat flakes, and Mauri-Pan-dried bakers yeasts (AB Mauri, Balakong, Malaysia). The chambers were placed into Secador desiccators (Bel-Art Products, Pequannock, NJ) with saturated solution of KCl (85% relative humidity) and kept at 25 ± 1°C in darkness. Before experiments, adult mites were transferred into Petri dishes with fungi. A total of 40 mites were introduced into each dish plated with one of the five fungi. Mites were cultivated at controlled conditions (22°C, 70% relative humidity in the room) up to 3 wk. The experiments were conducted in triplicate, and all results were approximately the same in each trial for each species of fungi.

Histology, including fluorescence microscopy, was the basic method used. Mites were fixed in modified Bouin–DuBosque–Brasil fluid (Smrž 1989) and embedded into Paraplast Plus (Sigma-Aldrich, Praha, Czech Republic). They were then sectioned on a Leica 2155 rotation microtome (Leica Camera, Wetzlar, Germany). Sections (5-µm thick) were stained in Masson's trichrome and observed using an AX-70 Provis microscope (Olympus, Tokyo, Japan) equipped with a Nomarski prism. The description of the gut is according to Smrž (1989).

All fungi were grazed, and subsequent to grazing various splotches of amorphous masses remained in the dishes. Those amorphous splotches could expand to cover an entire dish, depending upon the particular fungus. Those remnants or excrements were sampled from those plots, smeared onto microscopic slides, stained using orange G (Sigma-Aldrich), then observed under fluorescent light (Smrž 2002a, Smrž and Čatská 2010) using the same microscope.

As described elsewhere (Smrž 2000), chitinolytic enzyme activity was tested in mite homogenate on microscopic slides covered by a thin film of chitin and stained with basic fuchsine. The bacteria isolated from mite homogenate on methylphosphonic acid (Fluka, Sigma-Aldrich, St. Louis,

MO) were identified at the Czech Collection of Microorganisms Masaryk University Brno, Czech Republic an internationally certified laboratory.

The mites' population increase was estimated, although it ofen was difficult to establish accurate numbers due to the continuous movement of mites, and especially when they occurred in large numbers. The initial number (40) was known, and after 3 wk the numbers of mites were approximated for *A. alternata* and *P. claviforme*. More accurate numbers were determined for the lesser population abundances on *Mucor* sp., *Verticillium* sp., and especially *P. griseofulvum*. Therefore, only estimates were made for all dishes in the experiments (Smrž and Čatská 1987).

Results

Mucor sp

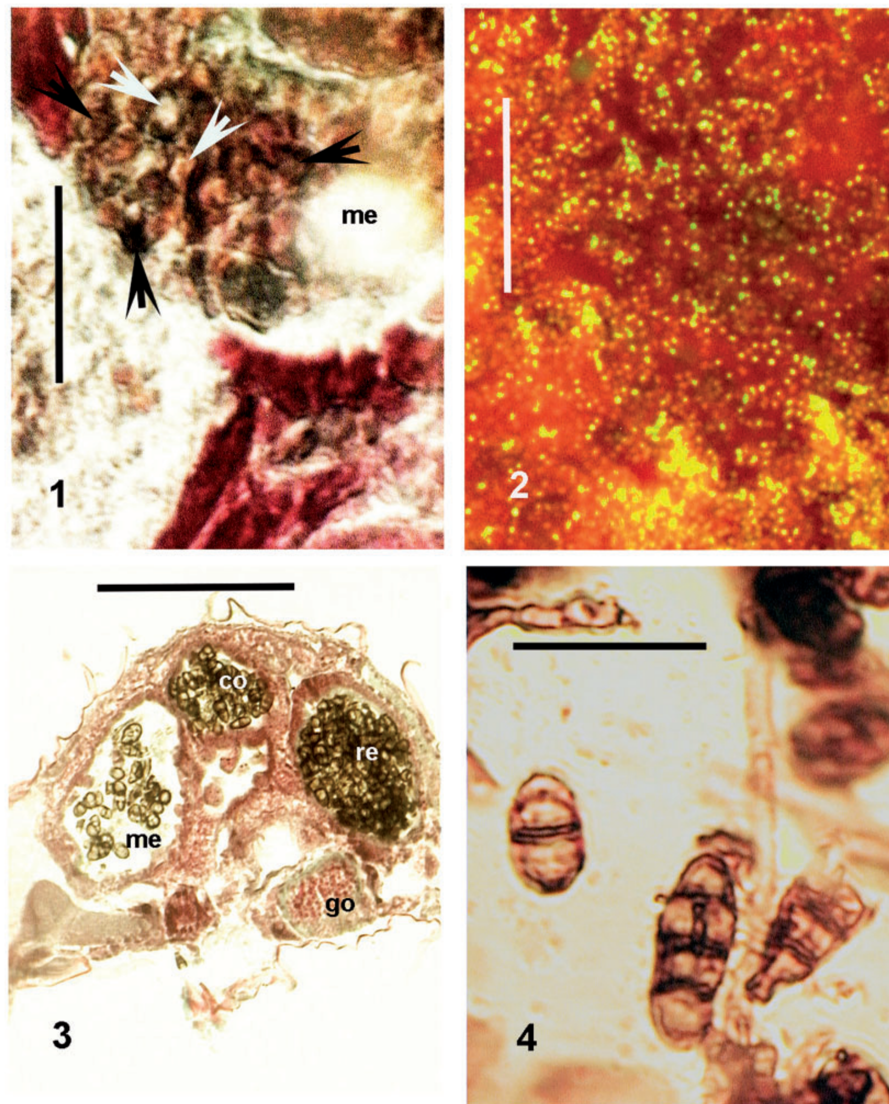
Many ingested spores were found in the mesenteron (Fig. 1), and there followed an amorphous mass in the rectum. On the other hand, destruction of the entire fungus including spores appeared to be very intensive and fluorescent light revealed only some amorphous mass and a huge number of bacteria in the excrement (Fig. 2). Neither spores nor their remnants were visible in those excrements. Chitinolytic activity was detected and the bacterium *Serratia marcescens* was isolated.

Alternaria alternata

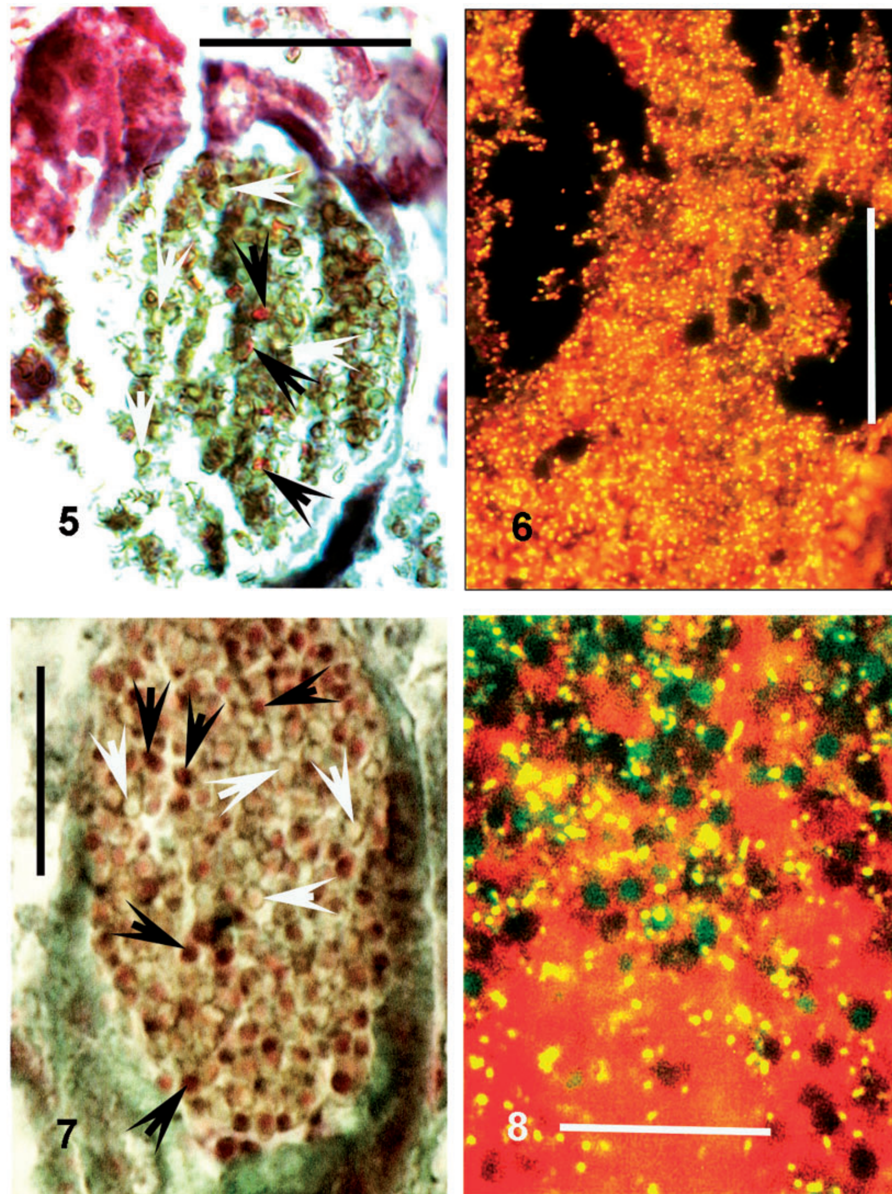
The histology revealed clusters of multi-cell spores of consumed fungus within the mites' gut. During passage through the gut, the spores became empty of any contents and mycelium appeared to be totally dissolved step by step caudad in the gut. The food bolus also exhibited conspicuous accumulation caudad up to the fecal pellet (Fig. 3). Excrement consisted mostly of rather empty spores (Fig. 4). Chitinolytic activity was detected and the bacterium *Brevundimonas vesicularis* was isolated.

Penicillium claviforme

Empty spores predominated in the gut over undigested spores (Fig. 5). The mycelium appeared to be totally digested without any traces. The majority



Figs. 1–4. 1—*Mucor* sp. as food: mesenteron, cluster of spores, horizontal section (black arrows point to intact spores; white arrows point to digested, empty spores); 2—*Mucor* sp. excrement filled with bacteria; 3—*A. alternata* as food: whole mite, parasagittal section; 4—*A. alternata*—excrement, fungal spores. Abbreviations used: co, colon; go, gonads; me, mesenteron; re, rectum. Masson's trichrome (1, 3), orange G, fluorescent light (2, 4). Scale bars: 100 μm (3), 20 μm (1, 2, 4).



Figs. 5–8. *P. claviforme* and *P. griseofulvum* as food: 5—mesenteron filled by *P. claviforme* spores, horizontal section, black arrows point to intact spores with contents; white arrows point to digested, empty spores; 6—*P. claviforme*—excrement filled with bacteria (points of light); 7—*P. griseofulvum* as food—rectum, horizontal section (black arrowheads point to intact spores; white arrowheads point to digested, empty, digested spores); 8—*P. griseofulvum*—excrement with intact spores (dark green or black round spots). Masson's trichrome (5, 7), orange G, fluorescent light (6, 8). Scale bars: 20 μm (5, 6, 7, 8).

of bacteria were found together with dead, digested spores (Fig. 6) in excrement. Chitinolytic activity was detected and *S. marcescens* was isolated.

Penicillium griseofulvum

Spores predominated in the gut (Fig. 7), but these were mostly all intact through the entire gut up to the rectum. The mycelium, however, appeared to be completely digested. Excrement was filled not only with huge amounts of bacteria, but also with undigested, living spores (Fig. 8). Chitinase activity was recorded and *S. marcescens* and *S. liquefaciens* were isolated.

Verticillium sp

Many fungal propagules were recorded throughout the gut. Spores occurred in the mesenteron (Fig. 9). Analysis of excrement revealed a mass of digested matter together with the bacteria and a small amount of undigested spores (Fig. 10). Chitinolytic activity was detected, and *Stenotrophomonas maltophilia* bacteria were isolated.

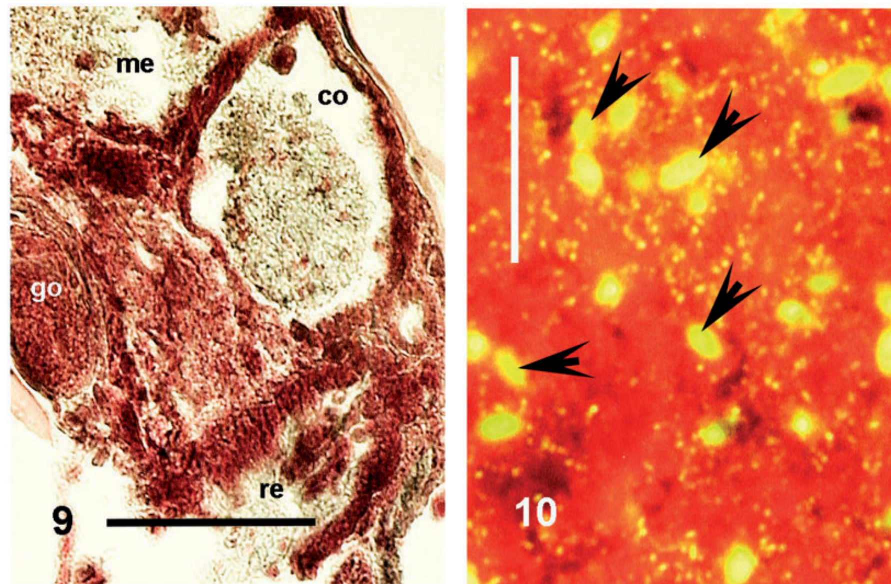
Ecological, rearing experiments

Mucor sp

Dark spots indicating fungus and especially spore content in the mesenteron were not so conspicuous on these mites' dorsa as was true in other cases (Fig. 11). The reproduction of mites was not as high as in the cases for other fungal species, no eggs were found on dish covers, and the mite population increased from 40 to ~400 individuals.

Alternaria alternata

Over 3 wk, the mite population dramatically increased from the initial 40 individuals to ~5,000 (Fig. 12), including huge numbers of both mites and oviposited eggs. As a result, their number can only



Figs. 9–10. *Verticillium* sp. as food: 9—hind gut; 10—excrement with intact spores (black arrows) and bacteria (points of light). Abbreviations used: co, colon; go, gonads; me, mesenteron; re, rectum. Masson's trichrome (9), orange G, fluorescent light (10). Scale bars: 20 μm (9, 10).

be approximated. Simultaneously, the fungus disappeared completely from the dish. All mites exhibited a dark spot on their dorsa, indicating the content of spores within the mesenteron.

Penicillium claviforme

The excrements formed in the dish a black, muddy mass together with fungal remnants (Fig. 13). The population increase was very great, but not so great as in the case of the aforementioned *A. alternata*. It increased from 40 to ∼800 individuals (again only estimated due to their large number and movement), but no oviposited eggs occurred on the dish cover. The mites exhibited a dark spot (from ingested fungus, mainly spores) on their dorsa (Fig. 13).

Penicillium griseofulvum

This fungus was grazed very poorly, and its cover was weakly destroyed in only a few places (Fig. 14). Mites exhibited dark spots on their dorsa. The mite population did not notably increase. Mite reproduction was not as high as seen in the cases of grazing the other fungal species, no eggs were found on dish covers, and the mite population grew from 40 to ∼100 individuals.

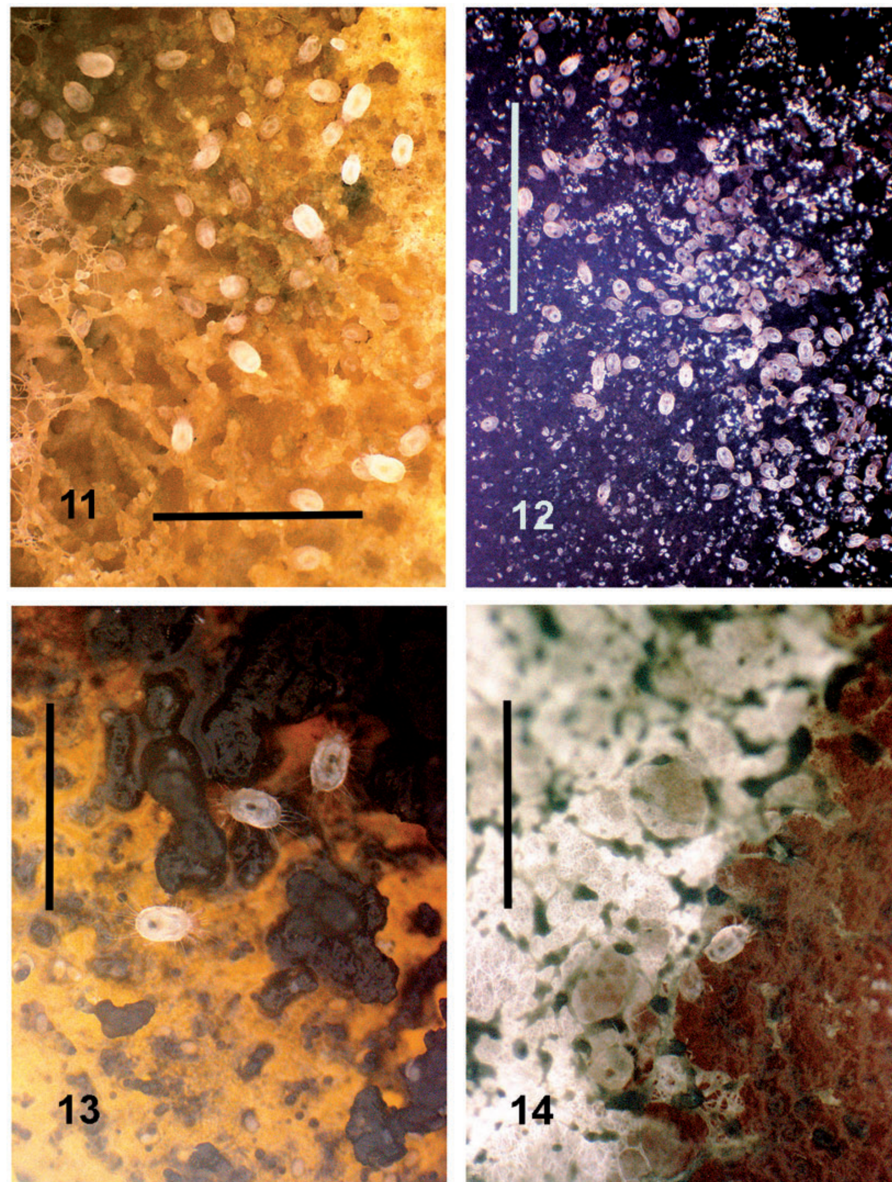
Verticillium sp

The dish's consumption was again patchy initially, but after 3 wk it was complete. Mite reproduction, however, was to a much lesser extent in comparison to *A. alternata* or *P. claviforme*. No dark spots were visible on the mite's dorsa, no eggs were found on dish covers, and the mite population increased from 40 to ∼400 individuals.

Discussion

The results obtained in this case study of *T. putrescentiae* feeding indicate that mycophagy is not a single and homogeneous category of nutritional biology, but rather that it is modified by specific symbiotic chitinolytic bacteria associated with mites and fungi (Table 1). The experiments yielded a comparison of digestion processes in one mycophagous mite species on different fungi under uniform conditions. In all cases, mite guts were filled in all parts. The mycelia were digested in the cases of all tested fungi, and therefore the presence of chitinolytic activity and isolation of chitinolytic bacteria are consistent with that phenomenon (Smrž 2003, Smrž and Čatská 2010). The chitinolytic enzymes did not appear to be necessary for digestion of the spore contents, although spore walls must be broken for digestion of the contents to occur. The spores were ruptured in a mechanical manner by the mites' chelicerae, to which the spores of *P. griseofulvum* appeared very resistant. The latter spores, therefore, passed through the gut intact up to the rectum and excrement without any disruption and digestion. Similar resistance was observed for *Verticillium* sp., albeit to a much lesser extent. Spores of other fungal species were disrupted and their contents extracted. The digestion of spore walls differed among the tested fungi, as well. No spore remnants were found in the rectum and excrement from *Mucor* sp. and only partially so for *Verticillium* sp. Although *A. alternata* and *P. claviforme* proved to be very advantageous foods, their spore walls were crushed but not digested. Therefore, spore skins were recorded in the rectum and excrement, albeit without any contents. The occurrence of intact spores was not unexpected in the excrement from *P. griseofulvum*, with its very resistant spore walls (Smrž and Čatská 1989). Different internal processes can be confirmed by such very simple characteristics as the color of dorsal spots on mites. Dark spots were consistent with dark spores (*Alternaria, Penicillium*). *Mucor* sp., however, formed dark spores as well, but mites exhibited only light dorsal spots. The spores of the latter fungus were digested and no skins were found in the excrement, which was in contrast to the other fungi mentioned above. A similar color was recorded for *Verticillium* sp., with its light but only partially digested spores.

The isolated bacteria have been confirmed as chitinolytic by several authors (*Serratia*: Brurberg et al. 2001; *Stenotrophomonas*: Zhang et al. 2001, Kobayashi et al. 2002; *Brevundimonas*: Jaspers and Overmann 2004, Kishore et al. 2005). In our experiments, *S. marcescens* dominated in the digestion of *P. claviforme*, *P. griseofulvum*, and *Mucor* sp. In these cases, the mycelia were digested in all fungi, as perhaps also were the spores in the case of *Mucor* sp. The participation of chitinolytic bacteria has been confirmed in several studies (Smrž et al. 1991, Smrž and Norton 1994, Smrž and



Figs. 11–14. Petri dishes with the mite population: 11—*Mucor* sp., 12—*A. alternata*, 13—*P. claviforme*, 14—*Penicillium griseofulvum*. Black mass = excrement, whitish mass = intact fungi, net of threads (14) = partially digested fungi. Scale bars: 0.5 mm (13, 14), 0.7 mm (11), 1 mm (12).

Table 1. Consumption and digestion of offered fungi and reproduction of *T. putrescentiae* feeding upon them

Fungus	Mite gut	Mycelium	Spore contents in excrements	Spore walls in excrements	Chitinolytic enzymes	Bacteria species	Final mite population	SD
Mucor sp.	Food in all parts	Digested	Digested	Digested	Present	*Serratia marcescens*	410.0	25.2
A. alternata	food in all parts	Digested	Digested	Undigested	Present	*B. vesicularis*	5.000*	Approx.
Penic.claviforme	food in all parts	Digested	Digested	Undigested	Present	*Serratia marcescens*	800*	Approx.
Penic.griseofulvum	food in all parts	Digested	Mostly undigested	Undigested	Present	*Serr.marcescens, Serr.liquefaciens*	106.1	17.2
Verticillium sp.	food in all parts	Digested	Partially digested	Partially digested	Present	*S. maltophilia*	402.2	36.7

**Mite populations are only approximated due to their large numbers and movement. SD = standard deviation.*

Trelová 1995, Smrž 2002a, 2003, 2009; Smrž and Čatská 2010). They form clusters adjacent to the mesenteron and mesenteric caeca not only in *Tyrophagus* mites, but also in mycophagous Oribatida (*Damaeus*, *Chamobates*, *Achipteria*: Smrž and Trelová 1995; *Archegozetes longisetosus*: Smrž and Norton 1994; *Belba*, *Damaeus*, *Metabelba*: Smrž and Čatská 2010). The source of such

bacteria remains at issue, but the difference among fungi consumed by a single mite species is able to indicate the somewhat different hyphoplane bacteria existing on fungal mycelia. It can also explain the shift of *T. putrescentiae* bacterial community after transfer of mites to a fungal diet (Hubert et al. 2012). The results suggest that different bacterial communities grow on different fungal species. This, however, should be the topic of additional studies.

The usefulness or digestibility of each fungus as food can be illustrated by the reproductive success of its consumers. Although *A. alternata* produces several toxins (Hudson 1986), the consumption of this fungus resulted in a population explosion for the tested mites. Grazing on the other fungi differed, but mite numbers increased on most fungi to a lesser extent relative to the start of the experiment. The exception, of course, is *P. griseofulvum*, which exhibited scarcely any digested spores.

It may therefore be concluded that the production of allochthonous chitinolytic enzymes was confirmed. Palatability and digestibility differ among fungi as a food source for a given mite species. The cause of these differences may be that there exist several types of chitin having different structures in the walls of the mycelia and spores of various fungi. It may be, too, that the binding sites on chitin with other substances (e.g., proteins) can play a substantial role (cf. Jollès and Muzzarelli 1999).

This article illustrates the usefulness of the applied method as a complement to other methods (Ruess et al. 2002; Schneider et al. 2004a,b). Histology is able to observe digestion in the animal gut, and the palatability of detailed food types can be estimated (cf. Maraun et al. 2011). The activity of the mite gut walls can be observed and the digestion of food simultaneously studied step by step from the ingested propagules in the mesenteron to the amorphous mass in the rectum or in excrement (Smrž 2002b). The plating of bacteria from mite homogenate supports such observations (Smrž and Čatská 2010).

Acknowledgments

This study was supported by project number GA15-09038S of the Czech Science Foundation (https://gacr.cz/en/).

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