Biological activity of the mite *Sancassania* sp. (Acari: Acaridae) from bat guano associated with the pathogenic fungus *Histoplasma capsulatum*

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Mites and the mammal pathogenic fungus Histoplasma capsulatum *are the major components of bat guano microbiota. Interactions between mites and* H. capsulatum *were evaluated under laboratory conditions. Acarid mites, mainly* Sancassania *sp., were the most abundant microarthropod in the sampled guano of the Mexican bat* Tadarida brasiliensis mexicana *and, based on its morphology,* Sancassania *sp. was similar to the cosmopolitan species* Sancassania sphaerogaster. *The mycophagous and vectoring activities of this mite were tested for* H. capsulatum *and two other fungal species,* Sporothrix schenckii *(pathogenic) and* Aspergillus sclerotiorum *(non-pathogenic).* S. *ca.* sphaerogaster *was able to reproduce in* H. capsulatum *and* S. schenckii *colonies, multiplying in great numbers under controlled fungal mycelial-phase culture conditions.* H. capsulatum *colonies were completely destroyed after 14 days of in vitro interaction with mites. In contrast,* S. *ca.* sphaerogaster *did not reproduce in* A. sclerotiorum *cultures.* S. *ca.* sphaerogaster *was found vectoring* H. capsulatum*, but not the two other fungal species studied.*

Key words: mites - *Sancassania* sp. - *Histoplasma capsulatum* - bat guano

Bat guano sustains a diverse microbiota, including insects, mites, bacteria and fungi. Among mites, the cohort Astigmatina (Suborder: Oribatida) includes mites inhabiting various terrestrial and aquatic habitats, often forming remarkable associations with their invertebrate and vertebrate hosts (Palacios-Vargas 2001). Most astigmatid mites from guano are involved in decomposing organic matter, such as the family Acaridae, including pests of stored products, cultivated and wild mushrooms and fungal colonies in laboratory collections (Hoffmann 1998). This family contains the ubiquitous genus *Sancassania*, which has also been reported as mycophagous and found in guano of different caves from Mexico, mainly located in the states of Morelos and Guerrero (Hoffmann et al. 1986).

The mycophagous activity of mites has been associated with contamination of fungal culture collections, as described for various genera of Astigmata (cohort: Astigmatina) and Prostigmata (Smith & Onions 1994); however, *Sancassania* has never been cited as a pest in micromycete culture collections. Several astigmatid mites show a broad food preference for macromycetes (Okabe & OConnor 2001, Klimov & OConnor 2003, Kheradmand et al. 2007). Acarid mites may also be important vectors of fungal spores in indoor mushroom commercial units (Okabe 1999).

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The ecological niche of the mammalian pathogenic fungus *Histoplasma capsulatum* var. *capsulatum* is related to naturally accumulated bird and bat guano. In this environment, the fungus grows as a mycelial-phase producing aerosolised infective forms (mainly microconidia and hyphal fragments) that, once inhaled by susceptible hosts, can produce an infection with a variable clinical course, including the systemic histoplasmosis disease (Tewari et al. 1998, Taylor et al. 2000). In general, the most frequent source of infection is found in bat guano located in enclosed places, such as grottos, caves, mines and abandoned buildings. There is a dynamic and complex food web in this habitat, where the "trophic levels" are occupied by different organisms in a food chain, including arthropods (such as mites) and fungi (such as *H. capsulatum*). To date, no interactions between mites and *H. capsulatum* in this unique environment have been recorded.

The aim of the present paper is to report the in vitro interactions between mites and the pathogenic fungus *H. capsulatum*, as both organisms share the same microhabitat (bat guano) of a particular ecological niche in a Mexican cave.

MATERIALS AND METHODS

Guano - Samples of *Tadarida brasiliensis mexicana* (Russell et al. 2005) guano were collected in the "La Boca" cave, located in the municipality of Santiago, in the state of Nuevo León, Mexico. This cave is a shelter for a colony of approximately 5 million of these insectivorous bats. Guano samples were collected at 30 m from the cave's entry. The temperature and relative humidity of the cave environment were 37°C and 70-80%, respectively. Guano samples were placed in different 100 mL plastic bottles (11 cm^3 of guano/sample) until filling each

one and the bottles were then transported to and kept in the laboratory under controlled conditions (darkness at 4°C without preservation solutions that could interfere with mites' survival) before processing. Ten samples from five well-defined sites in the cave were collected.

Mites - Mites were separated from the bat guano using Berlese funnels and placed on Petri dishes with a charcoal-plaster base. Then, mites were screened with a stereoscopic microscope Olympus SD30 (Olympus American Inc, US) and photographed with a Sony DSC-W70/SC E33 digital camera (Sony Corporation, China). Mite specimens were preserved in 70% alcohol and later mounted in Hoyer's solution, as described by Palacios-Vargas and Mejía-Recamier (2007). Mite gender and ontogenetic stage (adult, nymph and larvae) were determined by chaetotaxy and morphological characters in the laboratory. Mite identification was based on Lindquist et al. (2009). Species identification from the Acaridae family was conducted by Dr. Pavel B Klimov (Museum of Zoology, University of Michigan, MI, USA).

Live mites were maintained on charcoal-plaster Petri dishes incubated at 28°C in conditions of darkness and cultured in mycobiotic-agar (Bioxón, México DF) supplemented with commercial yeast (0.2 g) spread on the mycobiotic-agar surface, until use.

In vitro biological interaction between mites and fungi

Mycophagous activity - Three live mite specimens (2 females and 1 male), selected based on their abundance in guano samples, were placed on separate Petri dishes, each containing different fungal species previously reported in bat guano (Ulloa et al. 1998, 2006). Mycobiotic slant cultures of 4-week-old *H. capsulatum* (pathogenic) and two other micromycetes fungi, either 1-week-old *Sporothrix schenckii* (pathogenic) or *Aspergillus sclerotiorum* (non-pathogenic) were used for a set of triplicate assays. *H. capsulatum* strain (EH-53) was isolated from a patient infected in a cave in the state of Hidalgo, Mexico. It belongs to the "*Histoplasma capsulatum* Strain Collection of the Fungal Immunology Laboratory of the Department of Microbiology-Parasitology, Faculty of Medicine, National Autonomous University of Mexico", which is registered in the World Data Centre for Microorganisms database with the acronym LIH-UNAM WDCM817 (http://histoplas-mex.unam.mx).

Mites placed on sterile mycobiotic-agar, water-agar and potato-agar (Bioxón) served as a control without fungal cultures. These cultures were observed with a stereoscopic microscope every three days to record mite life cycles and every week to register their abundance. The mites were photographed with a digital camera (Sony) and were counted in four optical fields. The means of triplicate assays were recorded. The mycophagous activity of mites was documented using a digital video camera Sony Handycam DCR-PC109 (Sony) adapted to a stereoscopic microscope Zeiss Stemi SV6 MC 80 DX (Zeiss, Oberkochen, Germany).

Vectoring activity - To document possible fungal phoresy on mite species, mites were harvested individually from each culture of pathogenic fungi, where they were able to develop after 2-3 weeks of incubation at 28°C. Two types of assays, involving four mite specimens for each replicate, were conducted: (i) mites were decapitated and immediately placed on mycobiotic slants and (ii) before killing, mites were washed for 30 s with three different cleaning solutions (10% chloride, 70% ethanol and sterile distilled water) to eliminate fungal structures on the mite cuticle and then placed on mycobiotic slants.

All procedures were performed under sterile conditions. During incubation (2-4 weeks at 28°C), mycobiotic slants were observed daily to detect fungal growth. Colonies compatible with fungal morphotypes associated with the mite inoculum were documented after their cloning in mycobiotic slants and identification by macro and micromorphology. Each assay was performed in triplicate.

RESULTS

Mite abundance in guano - One thousand three hundred eighty-seven microarthropods, representing 21 taxa belonging to Arachnida, Acari (mites), Collembola and Insecta, were found. Mites were the most diverse and included 14 morphospecies with 1,365 specimens in four orders: Sarcoptiformes [Astigmatina (72.2%) and Brachypylina (4.2%)], Ixodida [Ixodoidea (0.2%)], Mesostigmata [Monogynaspida (5.4%)] and Trombidiformes [Prostigmata (18%)]. Collembola included four morphospecies with 12 specimens belonging to three orders: Entomobryomorpha, Poduromorpha and Neelipleona. Insecta included two morphospecies with nine specimens in two orders: Coleoptera and Diptera. Pseudoescorpionida was the less abundant taxon, with one morphospecies with only one specimen. Astigmatina predominated in the guano samples, emphasising that *Sancassania* sp. (57% of all microarthropod specimens collected) was the most abundant.

From the microarthropods collected, only two orders, Mesostigmata and Sarcoptiformes, were maintained alive in the laboratory. Two species of Mesostigmata (one and two specimens each) and two of Sarcoptiformes, which consisted of one species of the cohort Brachypylina (with one specimen) and one species of the cohort Astigmatina (with six specimens of the *Sancassania* sp.) were processed for in vitro biological interaction assays with fungi.

Interactions between mites and fungi - Before assessing in vitro biological interactions between mites and fungi, two species of Mesostigmata, one of Brachypylina and one of Astigmatina, were reared in separate plates containing mycobiotic medium supplemented with commercial yeast (see Materials and Methods).

Mite development on colonies of *H. capsulatum* or *S. schenckii* was monitored for 5-25 days. The presence of adults, nymphs and larvae was detected only on plates containing the *Sancassania* sp. (Astigmatina), indicating that reproduction of the mite took place. Fig. 1A-C shows *Sancassania* sp. growing on *H. capsulatum* and Fig. 1D-F on *S. schenckii* fungal cultures. Although a few Mesostigmata survived on *H. capsulatum* cultures for a short time, they were unable to reproduce and

eventually died, as did Brachypylina. Mites placed on cultures of *A. sclerotiorum* survived for 10 days and no ontogenetic stages were found (data not shown). Based on its morphology, the *Sancassania* species that was found to be associated with bat guano and reproduction on *H. capsulatum* or *S. schenckii* colonies is similar to the cosmopolitan species *Sancassania sphaerogaster* (Astigmatina: Acari: Acaridae).

Mycophagous activity of *S.* ca. *sphaerogaster* was well-demonstrated, as shown in a video animation (see details under Materials and Methods and Supplementary data). The absolute destruction of the *H. capsulatum* colony was observed after its infestation with *S.* ca. *sphaerogaster*. Alterations in colony macromorphology were visually detected. Three to 10 days after mite infestation, the border of fungal colonies presented remarkable changes with progressive destruction (Fig. 2A-C). After 14 days, these colonies were seriously damaged and destroyed by mite feeding activity. Regarding the *S. schenckii* colonies, destruction by the mites was less evident. In contrast, no mite reproduction occurred in other assays using *A. sclerotiorum* colonies and uncultured media (mycobiotic-agar, water-agar and potato-agar).

The maximum number of mites registered per assay at 12-13 days after fungal culture infestation was 80 on *H. capsulatum*, followed by 40 and 30 mites on *S. schenckii* mycobiotic-agar and potato-agar cultures, respectively.

The kinetics of mite abundance with regard to the infestation time on fungal cultures is shown in Fig. 3. The means of three sets of assay show that the highest abundance was found at days 12-13 on *H. capsulatum* mycobiotic-agar cultures, followed by day 18 on *S. schenckii* mycobiotic-agar cultures. In contrast, in the assays with *A. sclerotiorum*, the three mite specimens initially placed on the fungal cultures only remained alive for five days (Fig. 3).

In relation to the vectoring activity, out of 12 specimens of *S.* ca. *sphaerogaster* sampled from *H. capsulatum* cultures, only eight were associated with fungal colonies in the new culture plates (see details in Material and Methods). These colonies shared a macromorphology compatible with *H. capsulatum*. After cloning on fresh culture media, the fungal micromorphology was observed and the typical conidia of *H. capsulatum* were identified. Assays performed with *S. schenckii* were all negative. Assays performed with mites washed with different solutions (see Materials and Methods) showed no fungal growth after being observed for four weeks. In most cases, plates also exhibited bacterial contaminations.

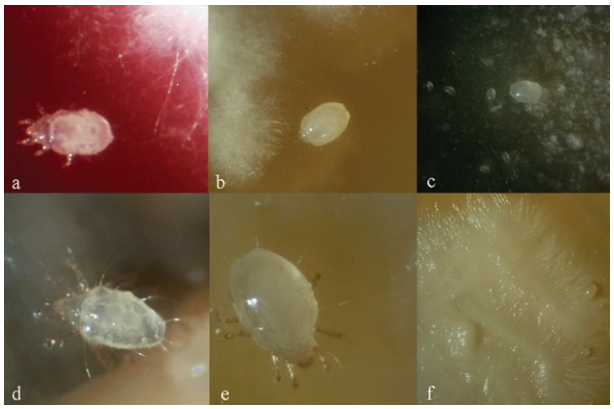

Fig. 1: adults, nymphs and larvae of mites grown on fungal plate cultures. a-c: *Sancassania* sp. grown on *Histoplasma capsulatum* culture: a: nymph; b: female; c: larvae, nymphs and females; d-f: *Sancassania* sp. grown on *Sporothrix schenckii* culture: d: male; e: female; f: adults.

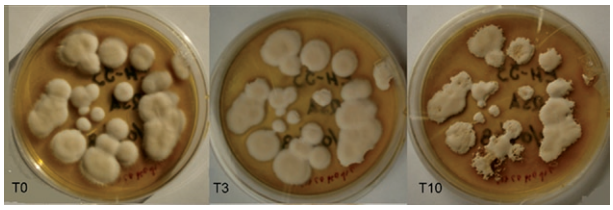

Fig. 2: macromorphological changes in *Histoplasma capsulatum* colonies infested with *Sancassania* ca. *sphaerogaster*. T0: immediately after infestation; T3: after three days; T10: after 10 days.

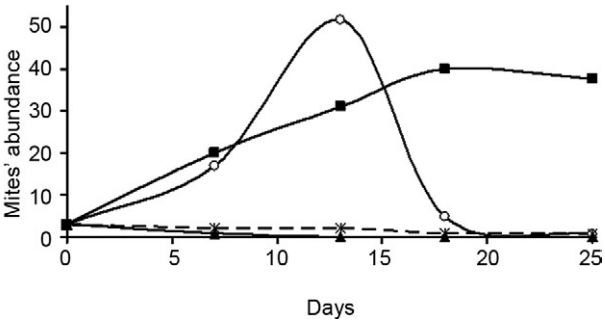

Fig. 3: abundance of *Sancassania* ca. *sphaerogaster* in different fungal cultures. Alive mites were placed in Petri dishes containing fungal species that had been cultured on mycobiotic slants. Mites were counted every three days. The plotted data represent the means of triplicate assays, until the 25th day (details under Materials and Methods). -○-: *Histoplasma capsulatum*; -■-: *Sporothrix schenckii*; -▲-: *Aspergillus sclerotiorum*; - x-: mycobiotic-agar (uncultured control).

DISCUSSION

S. ca. *sphaerogaster* was the most abundant mite in the guano from "La Boca" cave, representing 57% of all sampled microarthropods in this habitat. Some species of this genus have become specialised in feeding on basidiomycetes (Okabe & OConnor 2001, Klimov & OConnor 2003), while other species are generalists and may feed on a variety of substrates, including fungi (Kheradmand et al. 2007). Several genera of Acaridae are mycophagous and are reared on macromycetes culture (Okabe 1993, 1999) and some of them have even been reported to be pests of micromycetes: *Acarus siro*, *Histiogaster* sp., *Rizoglyphus robini, Rizoglyphus echinopus*, *Schwiebea* sp., *Tyrophagus putrescentiae* (Rivard 1961, Noble & Poe 1973, Yamada 1974, Price 1976, Okabe 1993, 1999, Smith & Onions 1994, Okabe & OConnor 2001).

In the "La Boca" cave environment, the mycobiota associated with bat guano could favour the development of *Sancassania* by providing the food necessary for its survival; however, no data were found showing that the diet of this mite is exclusively restricted to fungi. Most probably, *Sancassania* is a generalist in terms of food preference.

In the laboratory, the rearing of different species of Mesostigmata, Brachypylina and Astigmatina was attempted, using fungal culture as food source. However, only an Astigmatina, identified as *S.* ca. *sphaerogaster*, was able to reproduce. *Sancassania* poses serious challenges for identification due to its high morphological variability and the lack of a comprehensive review. To ensure *S.* ca. *sphaerogaster* species, sequences of the mitochondrial gene COI and the nuclear ribosomal gene 28SD9-10 will be processed.

S. ca. *sphaerogaster* developed its life cycle preferentially on *H. capsulatum* cultures and showed a maximum population size at 15 days after fungal culture infestation (Fig. 3), causing complete destruction of *H. capsulatum* colonies in 2-3 weeks. This suggests that the *H. capsulatum* mycelial-phase supplies the optimal requirements for *S.* ca. *sphaerogaster* development. Controls of uncultured media did not favour mite reproduction, suggesting that different medium components, by themselves, did not favour mite growth. *S.* ca. *sphaerogaster* is not specific for *H. capsulatum*, as it also grew on *S. schenckii* cultures. However, changes in the macromorphology of *S. schenckii* colonies were less remarkable, even though the number of mites remained constant for a long time, suggesting that mites consume this fungus in a non-preferential manner (Fig. 3). It is difficult to explain why *S.* ca. *sphaerogaster* was unable to reproduce on *A. sclerotiorum*. This could be because this fungal species does not provide the necessary dietary requirements for the mite, or some mycotoxins, known from *Aspergillus* (Lazo & Sierra 2008), might be involved.

The abundance of *S.* ca. *sphaerogaster* in bat guano indicates that this mite species could be considered a primary consumer in the guano food web. Based on the present findings, *S.* ca. *sphaerogaster* may be viewed as a potential natural biological control for *H. capsulatum,* since it shares the same environmental microhabitat and effectively destroys the fungus under laboratory conditions.

Vectoring activity mediated by *S.* ca. *sphaerogaster* was demonstrated for *H. capsulatum* cultures, indicating that a short-range dispersion of *H. capsulatum* may occur on *S.* ca. *sphaerogaster* in natural conditions. The mite cuticle is probably involved in this dispersal, since successive washes of mites using cleaning solutions eliminated fungal structures and, consequently, fungal growth was not detected. The absence of phoresy of *S. schenckii* on *S.* ca. *sphaerogaster* is probably related to the moistness of this fungal colony together with its leathery and wrinkled surface, which did not favour attachment of *S. schenckii* structures to the mite cuticle.

This is the first report on the interactions between the mite *S.* ca. *sphaerogaster* and the mammal pathogenic fungus *H. capsulatum*.

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