Report

Current Biology

A Brazilian Social Bee Must Cultivate Fungus to Survive

Highlights

- A social bee from Brazil must eat a fungus to survive
- The fungus grows inside brood cells over the larval food
- Fungus is transmitted to other generations via contaminated building materials
- This bee-fungus symbiosis shows parallels to fungus-farming insects

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In Brief

Menezes et al. report an obligatory relationship between a fungus and a social bee, in which the larvae eat the fungal hyphae that grows inside brood cells. The fungus occurs in the building material of the nest and uses larval food as growth medium. It is transmitted via swarming, suggesting this is the first case of a fungus-growing bee.

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A Brazilian Social Bee Must Cultivate Fungus to Survive

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SUMMARY

The nests of social insects provide suitable microenvironments for many microorganisms as they offer stable environmental conditions and a rich source of food [1–4]. Microorganisms in turn may provide several benefits to their hosts, such as nutrients and protection against pathogens [1, 4–6]. Several examples of symbiosis between social insects and microorganisms have been found in ants and termites. These symbioses have driven the evolution of complex behaviors and nest structures associated with the culturing of the symbiotic microorganisms [5, 7, 8]. However, while much is known about these relationships in many species of ants and termites, symbiotic relationships between microorganisms and social bees have been poorly explored [3, 4, 9, 10]. Here, we report the first case of an obligatory relationship between the Brazilian stingless bee *Scaptotrigona depilis* and a fungus of the genus *Monascus* (Ascomycotina). Fungal mycelia growing on the provisioned food inside the brood cell are eaten by the larva. Larvae reared in vitro on sterilized larval food supplemented with fungal mycelia had a much higher survival rate (76%) compared to larvae reared under identical conditions but without fungal mycelia (8% survival). The fungus was found to originate from the material from which the brood cells are made. Since the bees recycle and transport this material between nests, fungus would be transferred to newly built cells and also to newly founded nests. This is the first report of a fungus cultivation mutualism in a social bee.

RESULTS AND DISCUSSION

While carrying out research and beekeeping tasks on the stingless bee species *Scaptotrigona depilis*, we observed that the internal walls of the wax cells used to rear larvae were covered in a white fungus. To determine how general this was, we studied 30 colonies and found such fungal growth in all of them. Unlike honeybees, the brood cells of stingless bees are provisioned with a semi-liquid food mass that is regurgitated by nurse workers. The queen then lays an egg on the top of the food (Figure 1A), and then the workers close the brood cell, which is only opened when the adult bees emerge. Daily inspections of brood cells in these colonies showed that the fungus starts its proliferation when the egg is about to hatch, about 3 days after being laid (Figure 1B). It grows from the borders of the brood cell over the surface of larval food, toward the central area of the brood cell and to the top of the cell (Figure 1C). Fungal proliferation is intense until the third day of larval development (Figure 1D), after which the amount of fungus drops, and after a further 2 days, the fungus can no longer be seen in the brood cells (Figures 1E and 1F).

We identified the fungus using morphological and molecular tools, based on sequence analysis of the internal transcribed spacer (ITS) of the rRNA operon, ITS1-5.8S-ITS2 (Figure S1). The filamentous fungus showed 100% similarity with several species from the genus *Monascus*. The fungus is closely related to *M. ruber* (Genbank: AY498572) and *M. pilosus* (Genbank: AY498582), but further analyses are still needed.

To test whether the larva was actively eating the fungus, we recorded the behavior of 3-day-old larvae using time-lapse stereomicroscopy at one frame per second. The larva made circular movements around the brood cell and cut and ingested the fungal mycelia with its mandibles as they grew from the cell wall (Movie S1).

To test whether this symbiosis is obligatory, we carried out in vitro rearing of larvae (Table S1). Larval food was harvested from newly built brood cells, placed in acrylic brood cells, and sterilized with UV light. In half of the trials, the larval food was then re-inoculated with live fungal mycelia taken from brood cells. Newly hatched larvae were then transferred to the surface of the larval food. We repeated the experiment with five different colonies. In cells provided with fungus, 76% of the larvae survived (n = 150) while in cells without the fungus, only 8% survived (n = 150) (Figure 2) ($p < 0.0001$; Cochran-Mantel-Haenszel test; $M^2 = 144.400$; GL = 1). Larval food from cells without fungus

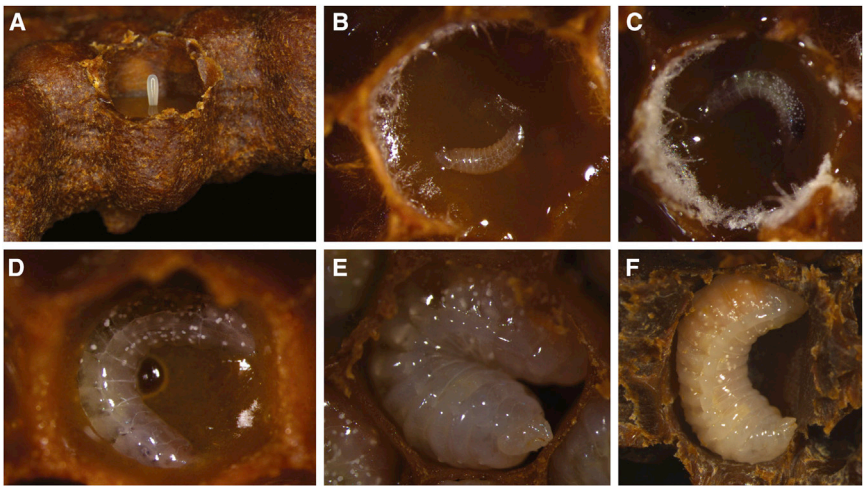

Figure 1. Growth and Consumption of Fungal Mycelia, *Monascus* sp., in Brood Cells of *Scaptotigona depilis*

Each cell is made of a mixture of wax and propolis called cerumen. Worker bees regurgitate brood food into a newly built cell, the queen lays an egg on the food, and the cell is sealed.

(A) Newly laid egg floating on the semi-liquid brood food; fungal mycelia not yet visible.

(B) 1-day old larva: fungal mycelia visible growing from cell wall onto larval food.

(C) 3-day-old larva: dense fungal mycelia on cell wall.

(D) 4-day-old larva: fungal mycelia have been eaten.

(E) 6-day-old larva: mycelia and brood food all eaten.

(F) Larva spinning cocoon.

smelled bad and showed other signs of spoilage such as stickiness. Larvae in these cells grew more slowly and by 6 days after transfer developed darkened guts and began dying in large numbers on day 7.

Since we only found mycelium structures in the colonies of *Scaptotrigona depilis* and never observed fungal conidia, we suspected that the bees propagate the fungus. It is probable that the fungus would stay alive in the bees' digestive tracts and thus be propagated through trophallaxis to other bees and to the provisioned larval food. To test this hypothesis and localize the source of the fungus, we tested 11 types of material from the nest of three colonies, including larval food from provisioned cells, crop content of nurse bees, body structures of bees, building materials of the nest, and honey and pollen reserves. We used larval food sterilized with UV light as a growth medium and re-contaminated it with these different materials to ascertain which would result in the reappearance of fungal mycelia (Table S2). The fungus was exclusively found in the cerumen (mixture of wax and plant resins used for building the brood cells, food pots, and all nest structures), not only from brood cells but from all nest parts that contained cerumen, including honey and pollen pots and the involucrum (cerumen layers covering the nest). However, fungal mycelia do not grow out from the cerumen until the cerumen is used to construct brood cells, where it had contact with liquid larval food and begins to proliferate rapidly. To observe details of the fungal growth, we visualized the cerumen of brood cells and fungus structures at different stages using a scanning electron microscope (Zeiss EVO 50). The fungus clearly originated from the cerumen as we predicted and formed complex filamentous structures (Figure 3).

Stingless bees do not reuse brood cells like honeybees, but they do recycle the cerumen from old brood cells to build new brood cells [11–13] (Figure S2). In this way, the workers can actively spread fungus to all cells in the colony.

In Meliponini bees, new nests are founded by swarming, with hundreds or thousands of workers and one or a few virgin queens [11–13]. Unlike honeybees, swarming in stingless bees is performed gradually and may take several weeks. The workers take building materials (mainly cerumen) from the mother nest and start building the structure of the new colony, such as the entrance, food pots, and involucrum. The workers then bring food from the mother nest to the new colony [11–13]. After the nest is ready, one or more virgin queens fly from the mother nest to this new nest [14]. Therefore, it is probable that the workers also actively propagate the fungus to the next generation, since stingless bees transport cerumen to daughter colonies during swarming. The swarming behavior of Meliponini, during which workers take building materials and stored food from the mother nest to the new nest, is unique among social insects [15]. This reproduction strategy of stingless bees represents a great opportunity for beneficial microorganisms and symbiotic relationships to be transferred vertically among generations and be "fixed" in the population. Pathogens can also be easily transferred vertically between nests, and therefore, a potent defense against pathogens is necessary if the nest material is reused.

This remarkable method of fungal propagation is very different to that of other fungus-growing insects. In the fungus-growing ants, ambrosia beetles, and a few species of termites (*Microtermes* genus and *Macrotermes bellicosus*) [16, 17], reproductive adults actively disperse their own fungus when they found new colonies, generally gathering spores in specialized storage structures for fungal transport [8, 18–20]. To our knowledge, our report is the first case of a swarming species thatpropagates fungus, and in this case, workers seem to be responsible for transmitting it to new colonies, not the reproductive adults.

The mutualistic *S. depilis-Monascus* sp. system entails several features that can be compared to other farming insects showing the highest levels of cultivation mutualism [1, 8]: (1) "habitual planting" or "inoculation" of specific fungal associates to appropriate substrates; in the bee-fungus system, inoculation is achieved by recycling the cerumen. (2) Cultivation aimed at the improvement of growth conditions for the crop; in the bee-fungus system, brood cells provide stable conditions and are filled with a semi-liquid larval food that seems to be essential for fungus growth, since they are present in several structures of the nest, such as involucrum and food pots, and do not grow at these places. (3) "Harvesting and consumption" of the fungal associates for food; bee larvae feed on the fungal mycelium.

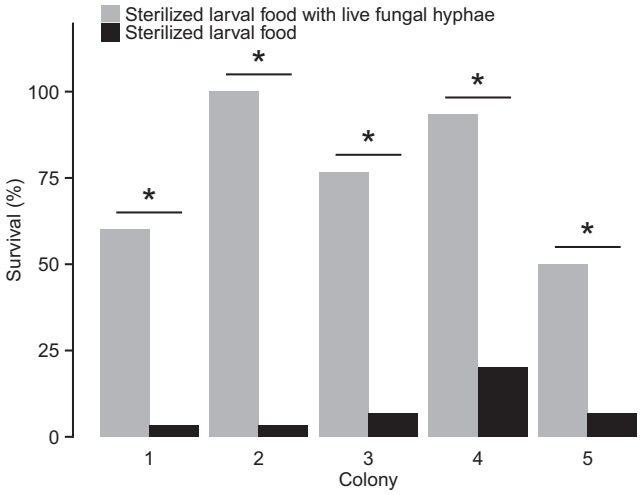

Figure 2. Survival of *Scaptotrigona depilis* Larvae Reared In Vitro
Black bars represent larvae fed with sterilized larval food, and gray bars represent larvae fed with sterilized larval food supplemented with fungal mycelia (*Monascus* sp.). We reared 30 larvae per colony, per treatment. Asterisks represent significant differences between treatments (Cochran-Mantel-Haenszel test, $p < 0.01$). See also Table S1 and Figure S3.

(4) Obligate nutritional dependency on the crop; bee larvae need to consume fungus to survive, although further research is necessary to determine the function of the fungus in the system, nutritive or protective.

A key difference to the known fungus-farming insects is that *S. depilis* do not tend their crop, past providing a suitable substrate. The system we describe resembles proto-farming animals that do not show active care for their crops, as in leaf-rolling weevil [21] and lizard beetles [22], a marine snail [23], gall midges [24], and a damselfish [25].

Although larvae may benefit from specific nutrients provided by the fungus, this remains to be adequately tested, and it does not seem likely that this is the main function of the fungus. The larval food is a rich source of nutrients and should be sufficient for the bee's requirements [26, 27]. If nutrition was the primary function of the fungus, we would not expect such a big difference in survival rate between the treatments in the in vitro rearing experiment, but we would expect smaller bees or deformed individuals in the treatment without fungal mycelia. This was not observed. While this does not exclude the possibility that *Monascus* is important for the production of a specific nutrient essential to larval development, this hypothesis begs the question of how other bees survive without mutualists.

An alternative hypothesis is that the fungus provides a protective function, like in several other symbiosis relationships [28]. Two facts suggest that the fungus produces secondary metabolites, which protect the larval food from microbial contaminations. In the in vitro rearing experiment, larval food without fungus smelled bad and showed other signs of spoilage. In addition, *Monascus* fungi are known to produce a variety of secondary metabolites [29–31]. Instead of a major nutritional benefit, it is possible that higher larval survival is due to secondary metabolites that keep the food free of pathogenic microbial contaminants. *Monascus*-fermented products have been widely used as a natural food coloring and preservative for meat and fish for over a thousand years in Southeast Asia [29–31]. Their secondary metabolites have also been used in traditional human medicine [30, 31]. Moreover, *Monascus*-fermented products show strong antibacterial and antifungal effects [29]. This suggests that *S. depilis* could be using the *Monascus* sp. fungus to protect their larval food from other microorganisms.

In order to test the effect of this fungus against other microorganisms, we performed a bioassay against *Escherichia coli* and *Staphylococcus aureus*. The test was performed using the disc diffusion method, on plate count agar (PCA) medium. A disc containing 50 units of Penicilin and 50 μl of Streptomycin was used as positive control, and a disc containing distilled water was used as negative control. We tested the growth inhibition effect of the following materials: new larval food, collected from cells containing eggs and no fungus; old larval food, collected from brood cells containing 3-day-old larvae; and fungus, collected from the borders of brood cells containing 3-day-old larvae. For each test, we used 15 μl of the pure isolate. We repeated the test nine times, and materials were collected from three different colonies. The fungus and the old larval food caused little or no growth inhibition against both bacteria tested (Figure S3), effects that were not significantly different from the negative control (Dunn's multiple comparisons test, $p > 0.05$). However, the new larval food caused a significantly larger growth inhibition effect compared to the negative controls (Dunn's multiple comparisons test, $p < 0.05$) and was not significantly different from the positive control (Dunn's multiple comparisons test, $p > 0.05$). This result neither rejects nor supports the hypothesis of an

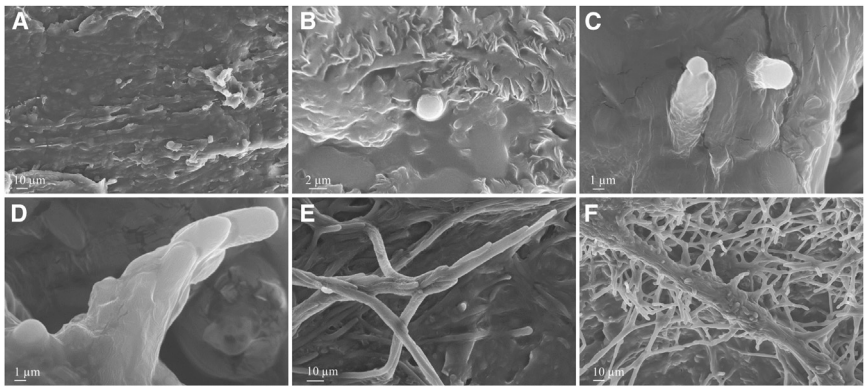

Figure 3. Scanning Electron Microscopy of Cerumen and Fungal Mycelia (*Monascus* sp.) from Brood Cell Cerumen Walls of *Scaptotrigona depilis*
(A–F) The fungus remains dormant until the egg hatches (A and B), grows from the cerumen of brood cells when the larvae is 1 day old (C and D), and produces complex filamentous structures that are eaten by the larvae (E and F). See also Table S2 and Figure S2.

anti-microbial effect of the *Monascus* sp. It may have an effect on specific bee pathogens that can develop on the larval food of *S. depilis*. The fresh larval food without the fungus must provide a harsh environment for the development of *S. aureus* and *E. coli*, suggesting the existence of an anti-microbial system that may control development of undesirable microorganisms during earlier stages, before egg hatching. Such an effect may be caused by other microorganisms that are known to occur in the larval food [10, 32] or due to other antimicrobial products from the hive, such as honey, plant resins, wax, or bee secretions [27]. Aging of larval food causes changes in enzymes activity [33] that could in turn diminish antimicrobial activity, which may even control *Monascus* sp. development, since before larva hatching fungal development is rather slow. Extreme mycelium proliferation could be damaging for the egg.

Like other social bees, *S. depilis* amasses valuable food stores and has developmental stages that are vulnerable to pathogens and parasites [15]. Bees thus seem to benefit from microorganisms that preserve their stored food and protect them from other, harmful microorganisms [27, 34]. These beneficial microorganisms are transferred from one generation to the next, and, while associated with their hosts, they are provided a suitable microenvironment in which to live and reproduce [7, 35]. These recent findings have practical importance for bee conservation because several pesticides, especially fungicides and bactericides, may not have a direct impact on the bees themselves but may affect their symbionts and make them vulnerable to diseases [36].

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, three figures, two tables, and one movie and can be found with this article online at http://dx.doi.org/10.1016/j.cub.2015.09.028.

AUTHOR CONTRIBUTIONS

C.M. and A.V.-N. designed the study, collected data, analyzed the data, and wrote the paper. V.L.I.-F. was involved in the study design and wrote the paper. A.J.M. and D.Z. identified the fungus. I.C.F and A.D.L. did the antimicrobial bioassays. All authors discussed the results and commented on the manuscript.

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