

## Natural History Note

### Cleaner Mites: Sanitary Mutualism in the Miniature Ecosystem of Neotropical Bee Nests

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**ABSTRACT:** Cleaning symbioses represent classic models of mutualism, and some bee mites are thought to perform cleaning services for their hosts in exchange for suitable environments for reproduction and dispersal. These mutual benefits, however, have not been rigorously demonstrated. We tested the sanitary role of bee mites by correlating mite loads with fungal contamination in natural nests of *Megalopta genalis* and *Megalopta ecuadoria* and by experimentally manipulating mite loads in artificial cells with developing brood. Field observations revealed significant correlations between the presence of mites and the absence of fungi inside the brood cells, as well as between the absence of mites and increased bee mortality. Likewise, experimental brood cells with mites have fewer fungal colonies than do cells without mites. Field observations and experimental manipulations, therefore, provide clear evidence of the sanitary effect of mites in nests of *Megalopta* bees. This bee-mite association constitutes one of the few examples of terrestrial cleaning mutualisms.

**Keywords:** acari, bee, cleaning mutualism, *Megalopta genalis*, *Megalopta ecuadoria*, symbiosis.

#### Introduction

Cleaning symbioses represent classic models of mutualism (Becker and Grutter 2004; Östlund-Nilsson et al. 2005) in which individuals of one species obtain food by removing external parasites from those of another species. However, most examples are aquatic and restricted to fishes and shrimps (Becker and Grutter 2005). A commonly cited example of a terrestrial cleaning symbiosis involves birds that glean ectoparasites from large mammals, but empirical tests have shown that this relationship is more complicated and may not always be mutually beneficial (Weeks 2000). Mites associated with some stingless bees (*Trigona*) are thought to decrease larval mortality due to fungi, but this

has not been rigorously demonstrated (Flechtmann and Camargo 1974). Parasitic mites associated with the wasp *Allodynerus delphinalis* act as “bodyguards” and defend juvenile wasps from attacks by a hymenopteran parasitoid (Okabe and Makino 2008). Another example of mutualism is mites associated with burying beetles. These mites reduce the number of fly larvae that compete with the beetles for food, and a lower number of flies on the carcasses increases beetle fitness (Wilson 1983; Wilson and Knollenberg 1987). This symbiosis, however, fluctuates between commensalism and mutualism over time. Here, we experimentally demonstrate that an association between bees and mites is a cleaning mutualism, adding to only a handful of documented examples of this kind of association.

Nests of Hymenoptera (ants, bees, wasps) are prone to attack by microorganisms (Schmid-Hempel 1998). As a result, many species have evolved behavioral and chemical traits to protect their brood and food. Examples include *Philanthus* wasps wrapping food with postpharyngeal gland secretions that reduce the amount of mold growing on the paralyzed prey (Herzner and Strohm 2007); wood ants furnishing nests with plant resins, rich in secondary compounds (Chapuisat et al. 2007); and fungus-growing ants producing metapleural gland secretions that decrease the amount of pathogenic fungi in the fungus garden (Fernández-Marín et al. 2006). Some species have evolved symbiotic relationships with other organisms that function to protect themselves from parasitic attack, such as the antibiotic-producing bacteria associated with the beewolf *Philanthus triangulum* (Kaltenpoth et al. 2005) or with fungus-growing ants (Currie et al. 1999; Mueller et al. 2005).

Both solitary and social bees construct brood cells within their nests (Michener 1974; Wcislo 2000), which create distinctive microenvironments (sensu Odling-Smee et al. 2003). Each cell is a miniature ecosystem, and the stored food sustains not only the development of the bee

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brood but also an extensive array of commensal and parasitic organisms, such as other arthropods, fungi, bacteria, and nematodes. Mites are one of the more common arthropod symbionts that live inside nests of bees, ants, and wasps (Arachnida: Acari; Eickwort 1990, 1993).

Bee-mite associations encompass a broad spectrum of interspecific interactions. Some mites are parasites; some are predators on other arthropods, including other mites; and others are hypothesized to be mutualists. One kind of mutualism may involve the cleaning services of mites by suppressing or curbing fungal growth within nests; hosts, in exchange, provide a suitable environment for mite reproduction, as well as an effective dispersal service (mites ride on the dorsal part of the bee's metasoma or mesosoma; Ordway 1964; Eickwort 1979). The associated sanitary benefits, however, have not been rigorously demonstrated until now (Flechtmann and Camargo 1974; see "Discussion"). This mutualism hypothesis makes two critical predictions. First, if cells contain mites, the incidence of contaminant fungal growth should be lower compared to that of cells without mites. Second, the bee brood should be healthier when mites are present in a cell, and brood survivorship therefore should be higher if raised in the presence of mites, compared to a mite-free brood. We tested these two predictions by correlating natural mite loads with fungal contamination of cells in natural nests of *Megalopta genalis* and *Megalopta ecuadoria* and by experimentally manipulating mite load in artificial cells with developing brood.

#### Overview of Bee-Mite Natural History

*Megalopta* Smith 1853 is a Neotropical genus of halictid bees (Moure and Hurd 1987) that exhibit a continuous spectrum from solitary to social life cycles and forage under dim light (Wcislo et al. 2004). Nests are usually constructed in dead wood and consist of a single excavated tunnel with individual cells made of chewed wood (fig. A1 in the online edition of the *American Naturalist*; see Sakagami and Michener 1962; Wcislo et al. 2004). Cells are provisioned with a mass formed from nectar and pollen. Following oviposition, the cell is sealed, and brood development from egg to adult eclosion takes about 35 days. Fungi are common in bee nests, given the nutritious substrate available (Batra 1965; Batra et al. 1973; Inglis et al. 1993; Hajek and Stleger 1994).

In *Megalopta*, a tight synchrony between bee and mite ontogeny exists: each developmental stage (stadium) of the host bee is often associated with a specific mite stage (N. B. Biani and W. T. Wcislo, unpublished data). When callow bees emerge, 91% of the mites are deutonymphs, the dispersal stage of certain mites (N. B. Biani, personal observation; see fig. A2 in the online edition of the *Amer-*

*ican Naturalist*). A preliminary identification of the tritonymphs (i.e., the penultimate mite stage before adulthood) collected from *Megalopta* nests places them in the genus *Laelaspoides* (Mesostigmata; H. Komplen, personal communication), which is known to be obligate bee mites associated with other closely related halictid genera, *Au-gochlorella* and *Caenaugochlora* (Eickwort 1966, 1979).

#### Material and Methods

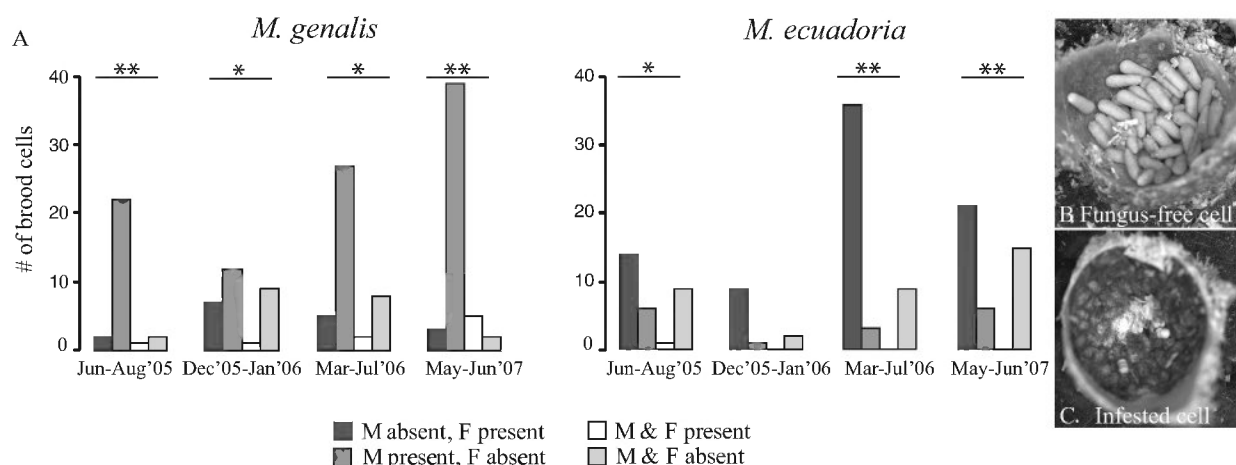
##### *Nests Collection and Field Surveys*

Nests were collected in Soberanía National Park, Barro Colorado Natural Monument, San Lorenzo Protected Area, and Bocas del Toro Province (Republic of Panama), during June–August 2005, December 2005–January 2006, March–July 2006, and May–June 2007. Throughout these field seasons, a total of 294 nests were collected, opened in the lab, and scanned under a light microscope for the presence of mites and fungi (39 nests were not included in this study because they were either abandoned or without brood, leaving 255 nests for analysis).

##### *Experimental Brood Cell Procedures*

Nests were opened in the lab, and the contents of natural cells were transferred into artificial brood cells made of paraffin, cover slips, and modeling clay (fig. A3 in the online edition of the *American Naturalist*). In order to empirically test the hypothesis that the presence of mites reduces fungal infection, we experimentally manipulated the presence/absence of mites in artificial brood cells. Mites were removed or added with a fine paintbrush, and all cells were opened and exposed for the same amount of time, even in control cells (sham treatment). The paintbrush was disinfected before each manipulation. Between seven and 10 tritonymphs were transferred to the artificial cells. No cells were experimentally inoculated with fungi, and we relied on natural rates of brood cell contamination.

For the experimental manipulations, we collected 27 nests that had mites and 23 nests that did not have mites. We then divided the group of nests with mites into two experimental groups: one was used as a control ( $N = 10$ ; sham opened but otherwise not manipulated), and the other one had mites removed ( $N = 17$ ). Similarly, the group of nests that did not have mites was divided in two experimental groups: one was used as a control ( $N = 12$ ; sham opened but otherwise not manipulated), and one had mites added ( $N = 11$ ). One independent variable was the presence/absence of mites at the time of collecting. The second critical, independent variable was the experimentally controlled presence/absence of mites at the end of the experiment.



**Figure 1:** A, Frequency distribution of brood cells with or without mites (*M*) or fungi (*F*) in *Megalopta genalis* and *Megalopta ecuadoria* for all four field seasons ( $\chi^2$ ; one asterisk,  $P < .05$ ; two asterisks,  $P < .001$ ). In *M. genalis*, mites are more abundant, and fungi are scarce, while in *M. ecuadoria*, mites are less abundant, and fungi are more pervasive. The negative relationship between these organisms holds for within-species associations. B, C, Brood cells with and without mites, respectively. Both cells are of approximately the same age. The pellets in the cells are feces deposited by the bee larvae. Cells with mites tend to be fungus free (B), whereas cells without mites show significant fungal growth (C) that may harm the developing bee brood. Cell diameters are on average 8 mm.

#### Assessing Levels of Fungi Contamination with Colony-Forming Units Procedure

Bees were reared in artificial brood cells, and after 10 days (approximately a third of the bee life cycle) fungal growth was quantified by plating diluted cell content and counting the number of colony-forming units (CFUs; Smith 1980; Christe et al. 2003). This method consists of diluting and homogenizing a sample (in this case, 0.01 g of pollen mass or bee feces) in 10 mL of a solution with 0.85% sodium chloride, 0.2% peptone, and 0.05% Tween 80 (a surfactant that facilitates the suspension of fungi spores in water; Sigma, St. Louis). Successive dilutions were made, and then an aliquot of 100  $\mu$ L was plated on a potato dextrose agar medium with 0.2 g/L of chloramphenicol as an antibiotic. The optimal dilution is the one that allows the growth of 30–300 colonies per plate; we used the dilution  $1 \times 10^{-4}$  for all the samples. All the cultures were done under a sterile laminar-flow hood. Fungi were allowed to grow in a humidity- and temperature-controlled room, and then the number of CFUs was counted blindly with respect to treatment. Two replicates were made for each dilution per sample, and the CFU counts were averaged. The CFUs of cells that received the same treatment and that belonged to the same nest were averaged in order to control for within-nest correlations.

#### Statistical Analysis

All data analyses were performed using SPSS 15.0 for Windows (SPSS 2006). Means are given with their standard

errors. For the field survey, a  $\chi^2$  test was used. The effect of mites in the experimental brood cells was tested with a two-way ANOVA. The data met all the respective assumptions for these two tests. Voucher specimens of the mites and bees are deposited in the Museo Fairchild, Universidad de Panamá, and the Dry Reference Collection of the Smithsonian Tropical Research Institute.

## Results

### Field Observations

Nests of *Megalopta genalis* and *Megalopta ecuadoria* were often colonized by mites carried by bees (*M. genalis*, 63%,  $N = 88$ ; *M. ecuadoria*, 17%,  $N = 79$ ). The number of mites per cell depended on the instar of both bee brood and mites, but it ranged from 0 to 25, with an average of  $8.3 \pm 5.8$  and  $N = 35$  tritonymphs per cell. Mites were more common in nests of *M. genalis* than in nests of *M. ecuadoria* (fig. 1A). During four field surveys in 2005–2007, spanning both wet and dry seasons, we observed a significant correlation between the presence of mites and the absence of fungi growing inside the brood cells of both *M. genalis* and *M. ecuadoria* nests (fig. 1). This provides support for the first prediction that the incidence of contaminant fungal growth is lower in cells with mites than in those without them.

There was no significant association between presence of mites and sex of brood ( $\chi^2 = 0.838$ ,  $P = .6577$ ,  $N = 25$ ;  $\chi^2 = 1.311$ ,  $P = .2522$ ,  $N = 13$ ;  $\chi^2 = 0.009$ ,

$P = .9229$ ,  $N = 97$ ; and  $\chi^2 = 1.543$ ,  $P = .2142$ ,  $N = 54$ , respectively, for the seasons mentioned in fig. 1A). Both male and female brood, therefore, become equally infested by mites.

Although most brood are successfully reared in the absence of mites, there was a significant association between healthy bees (i.e., bees that reach adulthood and are not infected by fungi) and the presence of mites ( $\chi^2 = 5.665$ ,  $P = .0173$ ,  $N = 283$ ). A total of 13 bees died out of 178 bees growing in the absence of mites, while only one pupa died out of 105 bees in association with mites (the nest with the dead pupa had been abandoned by the resident adults, and the brood cell was broken, so it is likely that other factors were responsible for the death of this pupa). This finding implies that the mites serve some sanitary function, supporting the second prediction of healthier bees when mites are present (fig. A1C, A1D).

#### Experimental Brood Cell Test

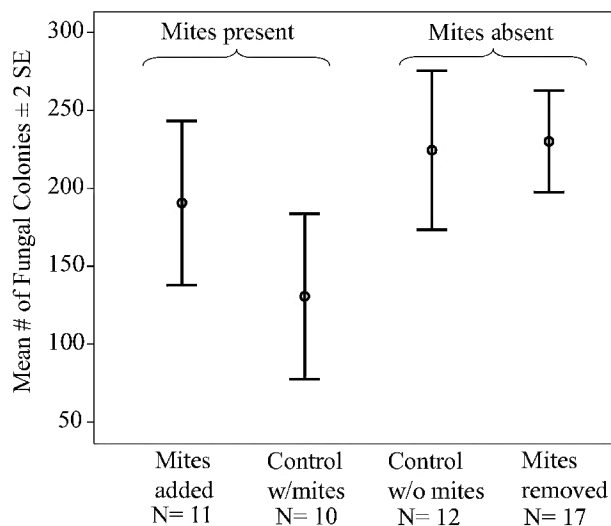
The effect of mites on the incidence of fungal growth in cells was tested by removing or adding mites to cells that naturally did or did not have mites, respectively. The control cells that naturally had mites throughout the experiment yielded an average of  $130.6 \pm 26.5$  CFUs, with  $N = 10$ . Cells that naturally did not have mites but had them added yielded an average of  $190.6 \pm 26.4$  CFUs, with  $N = 11$ . In contrast, cells that never had mites had an average of  $224.4 \pm 25.6$  CFUs, with  $N = 12$ , and cells that naturally had mites but had them removed had an average of  $230.1 \pm 16.3$  CFUs, with  $N = 17$  (fig. 2).

A two-way ANOVA assessed the effect of the presence of mites before and after experimental manipulation on the number of fungal colonies. Cells that always had mites or had mites added later had a significantly lower number of CFUs than do cells that never had mites or had mites removed ( $F_{1,46} = 8.227$ ,  $P = .006$ ). There was no significant effect on the CFUs counts due to the handling of the cells ( $F_{1,46} = 1.366$ ,  $P = .249$ ) and no interaction between the presence/absence of mites at the beginning of the experiment and at the end ( $F_{1,46} = 1.994$ ,  $P = .165$ ).

The effect size ( $\eta^2p$ ) is 0.152, which means that most of the variation can be explained by the treatments and that the size of the difference between means is moderate to large. The power is about 80%, and so it is reasonable to conclude that there are biologically real differences between the means.

#### Discussion

Our study provides the first rigorous demonstration of the repeatedly hypothesized cleaning mutualism between mites and bees. A single natural history account suggested



**Figure 2:** Means  $\pm$  2 SE of colony-forming units in the four experimental treatments. Control with mites are cells that naturally had mites. Cells that did not have mites received them experimentally. Control without mites are cells that naturally did not have mites throughout the experiment, and other cells had mites experimentally removed (two-way ANOVA:  $F_{1,46} = 8.227$ ,  $P = .006$ ).

that stingless bees benefit from the presence of mites inhabiting their nests because the latter clean the nest of fungal contaminants, but this account presented neither methodological details nor statistical analyses (Flechtmann and Camargo 1974). In contrast, our tests provide clear evidence of the cleaning effect of mites in nests of *Megalopta* bees. Laboratory results demonstrate that there are fewer fungal colonies (CFUs) in cells with mites than in cells without mites. This outcome mirrors the observational data from natural nests of both *Megalopta genalis* and *Megalopta ecuadoria*. Experimental manipulation of mite load further confirms that the absence or presence of mites is a causal factor in the growth of fungi (or not) in brood cells. It is unclear why *M. genalis* has generally higher mite loads compared to *M. ecuadoria*. This difference might be due to the incipience of the mutualism, or perhaps *M. genalis* spreads mites more effectively between nests. Future studies should address the ecological and species-specific reasons for the species differences in mite load between *M. genalis* and *M. ecuadoria*.

Symbiotic relationships range through a wide continuum from parasitic to mutualistic, as well as from diffuse to highly coevolved associations between pairs of species (Herre et al. 1999). Of these relationships, mutualisms are widespread, and some are thought to be one of the driving forces of diversification and stability in complex ecological systems (Boucher et al. 1982; Leigh 1991; Bronstein et al. 2006). The fact that not all the nests were colonized by

mites, and that brood can be reared successfully even in the absence of mites, may indicate that this bee-mite relationship is a facultative mutualism, at least from the bee perspective. However, as far as it is known, these mites are obligate bee associates (Eickwort 1966), and it remains unclear whether they can survive in favorable habitats outside of bee nests, such as flowers.

Host sex preference has evolved in some mites associated with hymenopterans (Cowan 1984; Hunter and Rosario 1988; Dawicke et al. 1992). For instance, some parasitic mites are found preferentially on female hosts, presumably to disperse to new nests, while others are found in males and are transferred to females during copulation (O'Connor 1982; Cowan 1984). Other bee mites do not exhibit such sex-based associations (Cross and Bohart 1969, 1992; O'Connor 1982; Houston 1987; Okabe and Makino 2003). Our results show that cells with male brood are as equally likely to have mites as are those cells that produce females. This prompts the question of the mode of transmission of the mites associated with *Megalopta*. Based on the ontogeny of the mites relative to that of the bees (N. B. Biani et al., unpublished data), we assume that bees are infested by mites in their natal nests during development. Nothing is known about how mites are transferred from males to females. Since only females potentially found new nests, mites developing in males' brood cells will constitute an evolutionary dead end unless there is a mechanism that allows their transference to females. The mating behavior of *Megalopta* is unknown, but venereal transmission during mating is possible, as occurs in mites associated with a eumenine wasp (Cooper 1955; Cowan 1984). Alternatively, both male and female *Megalopta* remain in the natal nest for up to 10 days after they eclose (Wcislo and Gonzalez 2006), a period that could constitute an opportunity for mites to transfer from males to females. Furthermore, *M. genalis* and *M. ecuadoria* are socially flexible species, meaning that some newly emerged females might disperse to found new nests, while others will remain in their natal nest to become part of a social unit. Hence, it is possible that mites attached to dispersing females will be developing a new generation in a brand-new nest, whereas mites attached to daughter bees remaining in their natal nest might colonize new brood cells in the same nest.

Mites are present in the nests of bees from diverse families, including Halictidae, Megachilidae, and Apidae; many taxa possess pouchlike structures (acarinaria) that localize mites to certain body regions, and museum specimens often house numerous mites (Batra 1965; Eickwort 1966, 1979, 1990, 1993; Cross and Bohart 1969, 1992; McGinley 1986; Houston 1987; Okabe and Makino 2002, 2005). Yet ecological and behavioral studies of bee-mite symbioses are scarce (Woodring 1973; Rack and Eickwort

1979; Houston 1987; Cross and Bohart 1992; Walter et al. 2002; Okabe and Makino 2008). Acarinaria were interpreted as structures that evolved to transport beneficial mites. However, due to the dearth of examples of such beneficial interactions, this notion has recently been challenged by an alternative hypothesis that the acarinarium is a defense mechanism against harmful mites because it decreases the likelihood that mites will leave any given host and thus infest other cells in the same nests (Klimov et al. 2007). The evolution of these morphological structures needs to be examined in more depth, for example, by exploring the percentage of species in which acarinarium are exclusively a female attribute (Okabe and Makino 2002). If bees are carrying harmful mites in the acarinarium, there would be strong sexual selection against males with these structures.

Coevolutionary interactions between wild bees and mites have been largely neglected, except for a few notable examples (Rack and Eickwort 1979; Houston 1987; Cross and Bohart 1992). Future studies need to investigate experimentally the nature of the relationships based on fitness advantages for each of the players, identify the mode of transmission and behavioral repertoires leading toward the association, and examine phylogenetic convergences or ecological constraints that may be affecting the system. The mechanisms that curb fungal growth in brood cells need to be further investigated, as our study did not address whether the mites are eating fungal spores or hyphae or whether they might be secreting biochemical substances with fungistatic activity. This study is the first experimental demonstration of the mutualistic nature of a bee-mite association and one of the few examples of terrestrial cleaning mutualisms.

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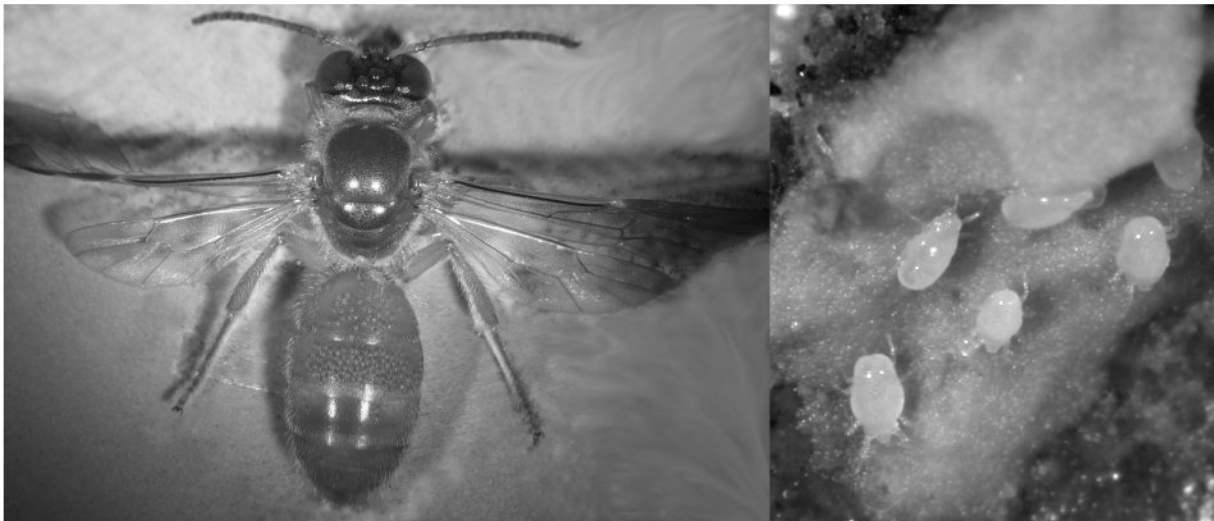
The Autoridad Nacional del Ambiente de la República de Panamá provided collecting and export permits. And, of course, we thank the bees and the mites.

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Left, *Megalopta* with two stages of mites. The tiny mites will remain attached to the bee and will eventually disperse into a new nest; the bigger mites will probably stay in their natal nest. Right, mites on pollen inside a brood cell. Photographs by Natalia Biani.