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Rhopalomastix is only the second ant genus known to live with armoured scale insects (Diaspididae)

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Abstract:

Rhopalomastix is a myrmicine ant distributed throughout tropical parts of Asia, with almost nothing known about its biology. Its closest relative *Melissotarsus* in Africa is the only ant genus known to live with diaspidid scale insects inside their nests, and to rely on these for food. Twelve colonies of four species of *Rhopalomastix* were sampled from seven tree genera in Singapore. Ants chewed tunnels under live bark, and these were occupied by many diaspidids belonging to five genera; at least two species are known to also live freely on the outside of trees. Inside ant nests, only a few diaspidids secreted their trademark shields. The association with the pupillarial *Fiorinia* is unexpected since they have fewer secretions that can be used as food by the ants. *Rhopalomastix* and *Melissotarsus* workers share several morphological adaptations, including powerful mandibles and silk glands, but while the legs of *Melissotarsus* are modified for traction during tunnelling, those of *Rhopalomastix* are not. Consequently, *Rhopalomastix* workers are able to walk outside their tunnels, but they behaved timidly except in *R. murphyi*. Silk was used to repair damaged tunnels, and spinning behaviour resembled that of *Melissotarsus.* Host trees do not gain protection from the ants against leaf herbivores, so they apparently get no benefits from this mutualism. We discuss the likely economic impact of this parasitic lifestyle, especially on fruit trees.

Keywords: mutualism, silk, *Melissotarsus, Andaspis, Fiorinia*, pupillarity, *Mangaspis, Pseudaulacaspis, Rhopalaspis, Aquilaria*

Introduction

Mutualisms between ants and scale insects are common in tropical rainforests on all continents (Heckroth et al. 1999; Blüthgen et al. 2006). Ants cannot feed on plant matter directly, but many species obtain honeydew from scale insects that extract plant fluids. In return, ants provide varying levels of protection and shelter for the scale insects. While some ant species merely guard and collect honeydew from scale insects scattered in the canopy (Buckley and Gullan 1991), other species share their nests with Pseudococcidae (mealybugs) or Coccidae (soft scales), e.g. *Dolichoderus* (Dill et al. 2002; Maschwitz and Hänel 1985) and *Crematogaster* (Heckroth et al. 1999; Ueda et al. 2008). Among scale insects, Diaspididae (armoured scale insects) is the largest and most specialized family with about 2700 species (García et al. 2018), but they are known to engage in mutualisms with only one ant genus (*Melissotarsus*) in Africa and Madagascar (reviewed in Peeters et al. 2017). Diaspidids differ from other scale insects because they do not excrete honeydew. Hence, ants tending diaspidids benefit by feeding on their protein-rich shield secretions and/or by eating a proportion of them.

Melissotarsus workers live in remarkable isolation from the outside world. The anomalous morphology of their head and legs represents extreme adaptations for tunnelling through live wood as well as silk spinning, that underlie an obligate mutualism with diaspidids (Fisher and Robertson 1999; Ben-Dov and Fisher 2010; Khalife et al. 2018). Indeed, workers are unable to walk outside their tunnels and must obtain all food from diaspidids. The Asian genus *Rhopalomastix* shows conspicuous morphological affinities with *Melissotarsus,* and a sister-genus relationship was confirmed in a molecular phylogeny of tribe Crematogastrini in subfamily Myrmicinae (Blaimer et al. 2018). However, almost nothing is known about its biology save for anecdotal notes dispersed among specimen labels and taxonomic descriptions. Wheeler (1929) reported that *Rhopalomastix rothneyi johorensis* nests in dead twigs of mango (*Mangifera indica*) and the bark of durian trees (*Durio zibethinus*), while *R. rothneyi javana* was collected from the bark of a dying mango tree. Over half the circumference of the tree up to man height was riddled with holes burrowed by ants, with numerous pupae seen. This suggests that *Rhopalomastix* may have similar nesting habits as *Melissotarsus*. It is corroborated by A. Manjikul's label information ("destroys bark of mango") for the holotype of *R. janeti* collected in Bangkok in 1936. Canopy fogging of lower canopy trees (average height = 19m) in Borneo (Floren and Linsenmair 1999) yielded two morphospecies of *Rhopalomastix*. An unnamed species from Vietnam nests in spaces under the bark of standing tree trunks (Eguchi et al. 2011). In the forests of Singapore, we show that *Rhopalomastix* ants live together with diaspidids inside tunnels chewed in live wood.

Methods

Study area and organisms

A total of 12 colonies of *Rhopalomastix* were sampled throughout Singapore (Table 1): Upper Thomson Nature Park (3 colonies, Oct 2016), Central Catchment Nature Reserve Mandai section (6 colonies, Nov 2016), Huogang Ave 3 (1 colony, Dec 2016), Pulau Tekukor island (1 colony, Dec 2016), Bukit Timah (1 colony, June 2017). GPS data are listed in Wang et al. (2018). We sampled the bark of trunks at man height to check the presence of ants, although workers were also observed on the outside of a few trees. Field images of nest architecture were taken. Small sections of wood about 8 x 10 cm were taken back to the laboratory for further observations.

In a separate survey, 4 baited (tuna and honey) pitfall traps (plastic cups 5 cm in diameter and depth) were attached (at man height) to the trunk of one *Durio zibethinus* tree in Upper Thomson Nature Park (Oct. 2016) for 48 hours before collection.

In the laboratory, behavioural observations were conducted immediately after removing the outer bark layer to expose ant tunnels. Multi-focused images were taken with Visionary Digital Passport System. Because diaspidids can never move once their stylets are inserted in plant tissue (during first instar), they cannot be transported by ants and thus our images give reliable information about their spatial distribution in the field. In contrast, ant brood can be moved around by workers. Attempts at rearing ants in the laboratory were unsuccessful, with ants dying out after 1 to 2 weeks.

Taxonomy of ants and diaspidids

Slices of wood with diaspidids attached were preserved in 70% ethanol. Specimens were slide-mounted after treatment in weakly heated 10 % potassium hydroxide, rinse in water and staining with fuchsin acid in a lactophenol solution, then immersion in a lavender oil extract and final mounting in Canada balsam. Slides were examined with a compound microscope (Supplementary Fig. S1). Adult females are required for taxonomy, and characters are mostly the appendages on the margin of pygidium (fused posterior segments), and the distribution of shield glands on dorsal and ventral surfaces (Miller and Davidson 2015).

The taxonomy of *Rhopalomastix* ants was resolved using morphological and genetic differences (Wang et al. 2018). Four new species were studied here: *Rhopalomastix glabricephala, R. johorensis* (stat. nov.), *R. murphyi* and *R. tenebra*. Voucher specimens for all twelve ant colonies are deposited in the Lee Kong Chien Natural History Museum, Singapore. Diaspidids are deposited in the Muséum National d'Histoire Naturelle, Paris.

Results

Rhopalomastix nesting biology

In Singapore, *Rhopalomastix* colonies were found in a variety of forest types ranging from native-dominated secondary forests, to abandoned plantations and even coastal forests (Table 1). Four species of *Rhopalomastix* inhabited seven genera of trees (*Aquilaria malaccensis, Artocarpus sp., Campnosperma auriculatum, Durio zibethinus, Fagraea fragrans, Macaranga gigantea, Mangifera sp.*) belonging to six taxonomic families. Recorded host trees ranged from small trees (>10cm diameter at breast height, DBH) to large trees with DBH of 1.1m. Host trees did not share obvious features, but bark texture and thickness probably affect suitability for the ants as well as the diaspidids.

All twelve ant colonies nested under the bark, together with large numbers of armoured scale insects (Diaspididae). Tunnels were restricted to the inner layer of bark (cork cambium) and did not extend into the secondary phloem (Fig. 1). The occurrence of ant tunnels can often be detected by lines of frass on the outside (Supplementary Fig. S2). In colonies A, B and L, a complex network of interconnected tunnels (Fig. 1) occupied a large area of the tree trunk up to man height, but in other trees, tunnels were apparently limited to small disjunct patches. In colony A, a total of 138 workers and 14 males were collected from tunnels in one small fragment of wood (depth 0.8cm, area 18.5cm^2, volume 14.8cm^3). *R. tenebra* workers (also winged males) were collected in trunk pitfall traps on the tree housing colony B, revealing that workers occasionally venture out of nests.

i. Evidence of chewing live wood

We noticed tunnels of constant diameters under the bark, at depths varying 1mm to 9mm from the outer bark layer (Supplementary Fig. S3). In colony A, ants were observed to exit the nests onto the bark surface through small holes sheltered by frass. After a small section of outer bark was removed to expose tunnels in colony H, workers and a dealate queen quickly retreated into intact tunnels. Next day, the exposed area had been closed off with frass. Similar deposits of frass were observed on the bark of all inhabited trees (Supplementary Fig. S2). We did not check if silk threads were present as a scaffold holding the frass together (see below). The presence of frass and extensive tunnels suggest that the ants actively chew through live wood.

ii. Behavioural response to disturbance

Transport of eggs, larvae and pupae away from exposed tunnels was recorded on multiple occasions (colony L in lab and others in the field). Upon disturbance, workers escaped to intact tunnels before a few reappeared to gather larvae, pupae and eggs. In some colonies, pupae and larvae occurred in dense aggregations in widened sections of tunnels, and they fell off easily after a tunnel was exposed.

Despite having a sting, workers of *Rhopalomastix johorensis, R. glabricephala* and *R. tenebra* did not defend themselves after we broke open their nests; they instead retreated and carried brood to safety. *Pheidole* sp., *Crematogaster sewardi* and *Tetramorium sp.* ants were repeatedly observed to quickly grab their pupae, larvae and even workers (Table 1). In contrast, *Rhopalomastix murphyi* workers behaved more aggressively following disturbance, even stinging humans. They were actively avoided by *Pheidole* sp., and only brood was stolen. These observations indicate how crucial is the protection provided by the host trees to *Rhopalomastix* workers and brood.

iii. Silk spinning behaviour

When tunnels were exposed in the field, thin filaments of silk appeared over the exposed area (Fig. 2). Silk originated from the head of *Rhopalomastix* workers, where the existence of more than 100 glandular cells releasing silk through ventral pores was recently documented by Johan Billen (unpublished). In colonies A and C, we observed a unique behaviour of continuous ‘head-bobbing’ along the edges of exposed tunnels (Supplemental Video). Silk filaments were drawn out from the ventral head region without involving the forelegs, unlike in *Melissotarsus*. A detailed sequence of silk use and mending of exposed tunnels was not recorded. In colony K and others, we noticed obvious lines of frass outside the trunk of host tree (Supplementary Fig. 2), and silk may function to aggregate the frass.

Mutualism with naked diaspidids

Armoured scale insects (Diaspididae) were found living inside the tunnels of all twelve *Rhopalomastix* colonies. Five diaspidid genera were identified across colonies, always occurring singly (Table 1). *Andaspis numerata, Pseudaulacaspis samoana, Rhopalaspis peetersi* (new genus and species) and *Mangaspis* sp. were associated with *R. johorensis*. *A. numerata* was also found with *Rhopalomastix murphyi* and *R. tenebra*. *Fiorinia sp.* occurred in *R. glabricephala* colonies.

First-instar nymphs ("crawlers") of diaspidids have legs and segmented antennae, and are the agents of dispersal. In free-living species, crawlers emerge from the shield of their mothers and move about the plant for several hours (unless they are wind-dispersed), seeking appropriate plant tissues to insert their sucking stylets. Female nymphs then start to secrete a hard shield and become sessile (legs and antennae degenerate), and they remain neotenic (in contrast, males develop wings inside their shields). Moulting occurs after about ten days and the exuvia is incorporated in the shield (as will the second instar exuvia). Females require only two moults to reach sexual maturity (Foldi 1990; Koteja 1990).

Inside the *Rhopalomastix* nests, female diaspidids were mostly naked, with only infrequent shields; the exuviae of first and second instars were embedded in these shields (Fig. 3). Loose exuviae were never found lying around. Males were found in only one genus, so reproduction is likely to be parthenogenetic. We observed crawlers coming out of a shield (colony I2, Supplementary Fig. S4) and walking independently (colonies B and I2).

When tunnels were exposed, ant workers hurriedly grabbed their brood and retreated into intact tunnels, but ignored the diaspidids. Since first and second instars as well as adult females cannot withdraw their stylets once inserted in plant tissue, they cannot be moved by the ants. We did not observe ants feeding or interacting with diaspidids.

The five different genera of diaspidids found inside *Rhopalomastix* nests are unrelated. Three genera have elongate female adults and shields (characteristic of subfamily Diaspidinae) while two genera are round in shape (subfamily Aspidiotinae). All instars lacked the trademark shield of Diaspididae, except for a few shields scattered among naked forms. The occurrence of *Fiorinia* sp. is intriguing since it shows pupillarial development (see below), so far unknown in ant-diaspidid mutualisms.

Andaspis numerata (Supplementary Fig. S1) (colonies A, B, J, L, N). This genus (tribe Diaspidini of subfamily Diaspidinae) is mainly Oriental. *A. numerata* is known from five plant families in India, Australia, Papua New Guinea, Fiji, Tonga and Western Samoa (García et al. 2018), and causes serious damage to branches of mature tea plants in India (Das 1976). In South Africa, *A. formicarum* was described from nests of *Melissotarsus* ants (Ben-Dov and Fisher 2010). We found a low density of female second instars and adults, as well as a few elongate shields (Fig. 3). One adult (winged) male was recovered in colony B.

Pseudaulacaspis samoana (colonies F, I, I2, O, Q) has affinities with *P. cockerelli* which is a polyphagous and cosmopolitan species. This is a huge pan-tropical genus in tribe Diaspidini of subfamily Diaspidinae. *P. samoana* was previously known only from coconuts in Fiji, Ryukyu islands, Tonga and Western Samoa (García et al. 2018). We found naked adult females and second instars that were widely spaced, and one shield containing an adult female (Supplementary Fig. S4).

Rhopalaspis peetersi (colony H) belongs to a new genus (subfamily Aspidiotinae) described by Matile-Ferrero and Foldi (2018). One colony of *Rhopalomastix johorensis* yielded dense aggregations of fully-grown adults, with second instars and eggs scattered throughout (Fig. 4 and Supplementary Fig. S5). When the tunnels were exposed, several eggs were seen halfway out of the mothers' bodies (Fig. 4). Eye spots in some eggs indicate that these were embryonated. This species is characterized by a concentration of glands on the last abdominal segments only, and very reduced marginal appendages.

Fiorinia sp. (colony E). Free-living adult females in this genus (tribe Diaspidini of subfamily Diaspidinae) are pupillarial: second instars grow considerably and adults develop inside their exuviae, which become heavily sclerotized and covered with a thin layer of wax. Wei et al. (2013) recorded 31 species from China. Inside tunnels of *Rhopalomastix glabricephala*, we found three extremely cryptic pupillarial scales (Fig. 5) on a narrow strip of wood (3 cm long), in addition to many second instars. A slide-mounted adult female (Fig. 6) revealed at least four crawlers within the second (pupillarial) exuvia. Ten mounted second instars revealed 6 females and 2 males.

Mangaspis sp. (colony K) belongs to a monospecific genus (subfamily Aspidiotinae) described from mango trees in India (Takagi and Kondo 1997); *M. bangalorensis* has affinities with our specimens. Only naked second instars and one damaged female adult were available for our study.

Discussion

Rhopalomastix construct their nests under the bark of living trees. This is the first definitive report that these ants can chew a network of tunnels that are inhabited by significant numbers of diaspidid scale insects. In 2010, S. Shattuck provided a clue of an association between *Rhopalomastix* and scale insects with an image of two yellowish bugs besides an ant pupa and a larva under bark in Danum Valley, Borneo. The five genera of diaspidids associated with *Rhopalomastix* in Singapore all share the remarkable characteristic that females can reach sexual maturity without secreting a hard wax shield. Naked adult females can produce offspring, either eggs or motile crawlers. Nonetheless, we found a few shields scattered among naked females of *Andaspis* (Fig. 3), *Fiorinia* (Fig. 5) and *Pseudaulacaspis* (Supplementary Fig. S4). Similarly in *Melissotarsus* nests, almost all *Morganella conspicua* instars including adults are naked, but rare shields occur away from aggregations (Peeters et al. 2017). Future studies need to confirm our impression that shields are less rare in *Rhopalomastix* nests. Except for *Rhopalaspis* females that were lying side by side, the naked diaspidids of other genera were less crowded than in *Melissotarsus* tunnels (see images in Peeters et al. 2017).

What benefits for *Rhopalomastix* ants?

The presence of diaspidids inside all nests examined is strong suggestion that they are a source of food for the ants. However, we lack direct observations of feeding or other interactions because ants stop normal activities and take cover as soon as we breached the tunnels. Most second instar and adult females lack a shield, but two pieces of evidence suggest that the glands used to secrete shields in free-living forms continue to be functional: (i) external morphology of females is normal with multiple gland openings on the pygidium; (ii) some shields are present inside tunnels. Hence the secretions (a mix of wax and proteins) are available as food for the ants (both larvae and adults), as discussed for *Melissotarsus* (Peeters et al. 2017).

Since the great majority of ant-associated female diaspidids reach sexual maturity without a shield, their discarded exuviae should be expected to lie around (in free-living forms, both first- and second-instar exuviae are integrated in the shields, and they were seen in the rare shields inside ant nests; Fig. 3). We never found such exuviae, so they are either eaten by the ants (just like ants eat exuviae of their own larvae), or discarded outside. In *Rhopalaspis peetersi*, the presence of second instars with only the basal part of shields (Supplementary Fig. S5) suggests that the first instar exuviae had been removed by the ants. *Fiorinia* adult females develop inside a pupillarium (hardened exuvia of second instar; Fig. 5) and a shield is not constructed even in free-living species; the wax glands are reduced in females (only few gland openings). Hence, we expect that *Rhopalomastix* cannot obtain much wax and protein secretions from *Fiorinia*, and predation of second instars may be the only trophic benefit for the ants.

Unlike *Melissotarsus* that have aberrant legs, *Rhopalomastix* are capable of walking outside their tunnels, and we cannot exclude that they can forage for dead arthropods or sweet secretions on the trunk of host trees. Moreover, *Rhopalomastix* workers have retained the sting, unlike *Melissotarsus*, and *R. murphyi* tried to repel other arboreal ants and aggressors that stole brood after we exposed their tunnels. At the first instance of disturbance, workers carried larvae and pupae to intact tunnels, and such brood caring behaviour does not occur in *Melissotarsus* (Peeters et al. 2017).

What benefits for diaspidids?

Diaspidids living with ants gain protection from weather and predation. The shields of free-living species create a safe microenvironment, but this becomes superfluous inside ant tunnels. Indeed, tunnels are cut off from the outside world and breaches are immediately repaired by ant workers. Diaspidids also gain easy access to parenchyma tissue that is rich with cell organelles. Ants chew tunnels in the cork cambium, and this helps diaspidids to insert their long stylets in secondary phloem (Fig. 1).

A majority of the dozen diaspidid species associated with *Melissotarsus* occur also as free-living forms on the outside of trees. However, a few species are known only as mutualists (Ben-Dov and Fisher 2010). Future fieldwork is needed in Singapore to determine which of the ant-associated diaspidids can also live freely. It is currently unknown what determines the preference of ants for only some diaspidids. In Singapore, three of the five genera of diaspidids living with *Rhopalomastix* belong to subfamily Diaspidinae (*Mangaspis* and *Rhopalaspis* are Aspidiotinae). In *Melissotarsus* however, diaspidids mostly belong to subfamily Aspidiotinae (Schneider et al. 2018). In Madagascar, just one species of *Melissotarsus* associates with five species belonging to Aspidiotinae (Ben-Dov and Fisher 2010).

Our sample size of twelve colonies (four species) does not allow recognition of a pattern of association across five diaspidid genera and six tree genera. While *Pseudaulacaspis samoana* lived in ant nests hosted by three species of trees, and *Andaspis numerata* in two tree species, *Fiorinia* sp. and *Rhopalaspis peetersi* were found only once (Table 1). The specificity of ant-diaspidid associations must be examined relative to the process of horizontal transmission. Peeters et al. (2017) discussed that a *Melissotarsus* founding queen is likely to determine suitability of host trees based on the presence of appropriate free-living diaspidids on the trunk; after she chews a shelter, crawlers can gather inside and are the nucleus of future aggregations. Similarly in *Rhopalomastix,* a degree of opportunism during colony foundation may explain the diversity of diaspidid partners.

Ability to chew tunnels in healthy wood

Despite anecdotal reports of *Rhopalomastix* nesting under the bark of trees, the ability to chew tunnels through live tissues was unknown. Indeed, many ants exploit tunnels already chewed in dead wood by the larvae of other insects. We documented the existence of tunnel networks in the cork cambium across different genera of host trees, with tunnels outlined by frass mixed with silk on the outside (Supplementary Fig. S2). Healthy wood has elastic fibres that are much harder to break than the brittle fibres of decayed dry wood (Aicher and Stapf 2016). It is expected that the enlarged head of *Rhopalomastix* workers houses huge closer mandible muscles and unusually large opener muscles, similar to *Melissotarsus* (Khalife et al. 2018).

Rhopalomastix workers have a specialized head as in *Melissotarsus,* but their legs retain a completely normal morphology. The striking leg modifications in *Melissotarsus* indicate an irreversible commitment to life in the confines of tunnels. The upward-oriented midlegs no longer allow walking on flat surfaces, but are instead used to gain traction against gallery walls during tunnelling. Stout tractor setae on the basitarsi of mid- and hind-legs grip the wood for added strength (this is not possible if resting on the flimsy terminal tarsi). Moreover, mid- and hind-legs have greatly enlarged coxae filled with powerful muscles, to push the whole body forward while the mandibles are chewing wood (Khalife et al. 2018). In contrast, *Rhopalomastix* have ordinary legs, they retain the sting and eyes are less reduced than in *Melissotarsus*. Unlike the other species, *Rhopalomastix murphyi* workers were observed walking on the bark surface, outside of their nest. Workers of *R. tenebra* were trapped in baited pitfall traps placed on the trunk. We do not know if *R. murphyi* or *R. tenebra* can supplement a diet based on diaspidids with yet unknown outside food.

Silk production

Rhopalomastix and *Melissotarsus* are the only ant genera in which adult females are able to synthetize silk. In contradiction to Hölldobler et al. (2014), J. Billen (unpublished) documented silk glands around the mouth region of *Rhopalomastix*. Silk is used to hold wood particles together in the roofs of tunnels, and is thus an essential component of nest defense. Spinning behaviour is elaborate in *Melissotarsus,* involving adaptations of the forelegs (Fisher and Robertson 1999). Tarsomeres on the forelegs are very hairy and used to draw out the silk filaments. The basitarsi are greatly enlarged and house a new gland that secretes a proteinaceous substance (J. Billen unpublished), suspected to give extra rigidity to the matrix of silk and wood. In contrast, *Rhopalomastix* forelegs lack enlarged basitarsi. In *R. murphyi*, head movements ('head-bobbing') are apparently enough for silk release and application to the substrate (Supplemental Video). Roofs of tunnels seem less stiff than those of *Melissotarsus,* and we speculate that they are more susceptible to intrusion by other ants and insects. Also noteworthy is the contrast in behaviours after we breached the tunnels: *Melissotarsus* workers immediately start to spin silk and repair (Peeters et al. 2017), while *Rhopalomastix* escape to safety.

Rhopalomastix and *Melissotarsus* differ from other ants nesting in live plant tissue. Species that inhabit specialized ant-plants (e.g., *Cecropia*, *Acacia*) occupy preformed nest cavities, and structural modifications seldom go beyond chewing entrance holes or removing soft parenchymous tissue (Bequaert 1922; Huxley and Cutler, 1991). *Gesomyrmex* ants remove parenchyma from the inside of small living branches where they nest, although an entrance tunnel must first be chewed through hard wood (Peeters et al. 2017). In cork oak trees infested by *Lasius* ants, these excavate tunnels in the cork bark to accommodate the giant aphids from which they obtain honeydew (Loi et al. 2012). In contrast, *Melissotarsus* and *Rhopalomastix* create their nests by tunnelling into live plant tissue. Together with powerful mandibles, silk production allows living plants to be used in a manner different to other ants.

Different evolutionary stages in a lineage specialized to feed indirectly on trees

Rhopalomastix and *Melissotarsus* are sister genera in Tribe Crematogastrini (Blaimer et al. 2018), and they exhibit a unique combination of morphological features (Bolton 1982). This lineage has engineered a novel ecological niche whereby they live permanently in live wood and feed on trees via the diaspidids. Many ant genera are engaged in mutualisms with scale insects, but only *Rhopalomastix* and *Melissotarsus* associate with Diaspididae, even though this family is the most speciose among scale insects. Bolton (1982) already noted that *Melissotarsus* workers have a more specialized morphology than in *Rhopalomastix.* Both have enlarged heads with powerful mandibles, but the original adaptations in mid- and hindlegs are restricted to *Melissotarsus* (Khalife et al. 2018). Both genera stand out among ants by their ability to produce silk, used for securing nests. However, only *Melissotarsus* has enlarged fore-basitarsus with exocrine secretions suspected to function in strengthening the silk covers. It remains unclear how these different degrees of morphological specialization affect colony characteristics. Colony size remains unstudied because we only sampled *Rhopalomastix* tunnels at man height. A high density of individuals (~10 workers / cm^3 of wood) was recorded but we did not determine how far the tunnel networks extend in the trees.

Impact on host trees

Ants, scale insects and host trees can be involved in triangular associations where all partners obtain benefits (Retuerto et al. 2004; Styrsky and Eubanks 2007). This is clearly not the case with *Rhopalomastix* since ants and brood were preyed upon by other arboreal ants and cannot protect host trees against leaf herbivores. Successful ant-scale insect mutualisms presumably impact tree health (Handler et al. 2007). *Melissotarsus*-infested trees suffer a theoretical cost since huge numbers of diaspidids feed on plant tissue, and a negative effect was shown in mango and other cultivated fruit trees (Mony et al. 2002). We found no obvious signs of stress in trees harbouring *Rhopalomastix,* and some had grown to a very large size. *Melissotarsus* colonies include up to a million ants (Mony et al. 2002), but we do not know if *Rhopalomastix* colonies are equally populous. The economic impact of *Rhopalomastix*, in fruit plantations especially, needs to be studied throughout their extensive distribution in Asia (Pakistan to eastern Australia).

The discovery of *Rhopalomastix* together with *Rhopalaspis* diaspidids in *Aquilaria malaccensis* trees is of particular significance. Several species of *Aquilaria* are highly valuable commercial trees (Akter et al. 2013) because of Agarwood, a fragrant resin used for perfumes, incense and traditional medicine. Agarwood (called 'Oud' in Arabic) is a complex defensive compound produced when the heartwood of older trees (>15 years) is invaded by an ascomycete mould (Liu et al. 2013). Considered as 'Vulnerable' in the IUCN Red List and World List of Threatened Trees (Oldfield et al. 1998), *Aquilaria* is over-exploited in many countries; distilled Agarwood oil can fetch up to US$50000 per kg. Current methods for inducing the production of Agarwood from *Aquilaria* rely on drilling holes in the trunk (Akter et al. 2013), and this may favour the establishment of ant colonies and their diaspidids. Alternatively, the tunnelling behaviour of *Rhopalomastix* might help to "heal" the wounds over time, i.e. if ants are already living under the bark, induction methods for Agarwood may be less effective. It is noteworthy that diaspidids living with *Rhopalomastix* in *Aquilaria* belong to a distinct new genus (*Rhopalaspis*). Fieldwork is required to determine the characteristics of free-living *Rhopalaspis* (if they exist), and whether they are exclusive to *Aquilaria* trees.

Conclusion

This is the first detailed report of nesting biology in *Rhopalomastix*, showing it to be the second ant genus to be associated with diaspidids, besides *Melissotarsus*. *Rhopalomastix* chews tunnels in live wood and forms non-specific mutualisms with different genera of diaspidids. Although we lack empirical evidence, this relationship is likely to be detrimental to host trees, especially in fruit plantations. Further studies must investigate the specificity of the associations between ants and diaspidids, and details of mutual benefits.

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Figure legends

Figure 1. *Rhopalomastix tenebra* workers (colony B) and *Andaspis numerata* attached in the cork cambium layer. Tunnels do not extend into the secondary phloem (white tissue) where long stylets allow diaspidids to feed. A worker carries a larva to safety.

Figure 2. *Rhopalomastix murphyi* workers (colony A) after the bark of host tree has been opened. Silk filaments can be seen (arrows). Photograph by Chui Shao Xiong

Figure 3. Workers and one larva (upper left) of *Rhopalomastix murphyi,* together with *Andaspis numerata* diaspidids inside galleries chewed under the bark (colony A). Four

shields (arrows) enclosing adult females are scattered among naked forms. Note the first and second instar exuviae incorporated in a shield (green circle). Photograph by Chui Shao Xiong

Figure 4. Dense aggregation of naked adult females and eggs of *Rhopalaspis peetersi* inside a tunnel of *Rhopalomastix johorensis* (colony H). Several individuals are in the process of ovipositing.

Figure 5. Three pupillarial scales (containing adult females) of *Fiorinia* sp. scattered among naked second instar females, in tunnels of *Rhopalomastix glabricephala* (colony E).

Figure 6. Mounted *Fiorinia* sp. from colony E. Unlike most Diaspididae, 'pupillarial' adult females develop and lay eggs within the enlarged exuvia (A) of the second instar. The now-shrunken body of the adult (a) is confined to the cephalic region; her tentorium (Tent) and stylets (Styl) are visible. The second instar's tentorium (B) is seen above 4-5 first instar nymphs (b) that can be recognized by their tentorium, segmented antennae (Ant), stylets and six legs (L). The pygidium of second instar (C) is here folded, so the marginal appendages are not visible. A split in the posterior region of second instar exuvia will allow the crawlers to emerge. Stacked image by Adam Khalife.

Electronic supplementary material

Figure S1. Mounted adult female of *Andaspis numerata* from a colony of *R. murphyi*, showing tentorium (Tent), stylets (Styl), pygidium (Pyg) and an embryo (Emb)

Figure S2. Surface of bark with tunnels outlined by wood shavings (frass) in colony K. Photograph by Chui Shao Xiong

Figure S3. Tunnels at different levels in the bark of *Durio zibethinus* (colony A) showing thickness, spatial arrangement and constant diameters of tunnels chewed by *Rhopalomastix*. Scattered diaspidids are visible. Bark surface is below.

Figure S4. Damaged shield of *Pseudaulacaspis samoana* showing eggs and crawlers (with antennae; red arrow) inside colony I2 (*Rhopalomastix johorensis*). Note an apparent inclusion (exuvia?, black arrow) in the shield.

Figure S5. Naked second instars with ventral layer of wax (green circles) in *Rhopalaspis peetersi.* First instar exuviae are missing in some, revealing they have been removed (eaten?) by the ants.

Supplemental Video. Stereotyped behaviour of 'head-bobbing' in *Rhopalomastix murphyi* (colony C). Silk is applied along the edges between exposed and intact tunnels.