Glandular innovations for a tunnelling life : Silk and associated leg glands in *Melissotarsus* and *Rhopalomastix* queen and worker ants

Johan Billen¹ and Christian Peeters²

¹ KU Leuven, Zoological Institute, Naamsestraat 59, box 2466, 3000 Leuven, Belgium.

E-mail: johan.billen@kuleuven.be

² Sorbonne Université, CNRS, Institut d'Écologie et des Sciences de l'Environnement, 75005 Paris, France. E-mail: christian.peeters@upmc.fr

Key-words: silk gland; Delage-Darchen gland; basitarsus; morphology; ultrastructure; caste dimorphism

Abstract

As in other Hymenoptera, adult ants cannot secrete silk, unlike the larvae that spin a cocoon prior to metamorphosis. Fisher and Robertson (1999) first showed the existence of a silk gland in the head of adult *Melissotarsus beccarii* workers, and we confirm this with detailed histology and ultrastructural comparisons of both queens and workers. This African genus exhibits extreme morphological adaptations (legs, head shape and mandibular muscles) for tunnelling behaviour inside living trees, that underlie an obligate mutualism with scale insects. *Rhopalomastix* is its sister genus distributed across Asia, and we show that queens and workers also have a silk gland. This lineage of minute workers relies on silk to secure their network of tunnels against other arboreal ants. We show striking differences between these genera in the anatomy and ultrastructure of the cells that secrete silk, especially numerous vacuoles and an unexpectedly branched end apparatus in *Melissotarsus*. Moreover, the legs of *Melissotarsus* are much more specialized for tunnelling, and this includes highly expanded basitarsi. The latter house the novel 'Delage-Darchen gland', and we document its anatomy and ultrastructure, suggesting a proteinaceous secretion to harden roofs made of silk combined with wood fragments. The restriction of the Delage-Darchen gland to *Melissotarsus*, combined with a modified silk gland (an almost three-fold increase in the number of secretory cells, and ultrastructural differences suggestive of higher secretory activity), are evidence of an outstanding evolutionary divergence relative to *Rhopalomastix*. Synthesis of silk by adults is a significant innovation among ants, but its augmented production in *Melissotarsus* makes them better adapted for the hazards of arboreal life.

1. Introduction

Insects have exocrine glands that fulfil essential communication and other functions. Gland diversity peaks in the social insects, with a variety of secretions that underlie the optimal

functioning of societies. A record number of exocrine glands are known in the ants (Billen and Šobotník, 2015), including several that are associated with shifts in lifestyles. Examples include fungus-growing ants that have glands to feed mutualistic bacteria that protect their fungus garden with antibiotics (Currie et al., 2006), social parasites that often rely on glandular secretions to enter colonies of their host species (Grasso et al., 2005), as well as a plethora of glands used to coordinate different types of cooperative foraging.

Melissotarsus and *Rhopalomastix* engineered a novel ecological niche: these minute ants (2-2.5 mm) live permanently in live wood and feed on diaspidid scale insects (Peeters et al., 2017 and references therein; Yong et al., 2019). The two genera group together on a separate branch within Tribe Crematogastrini (subfamily Myrmicinae) (Blaimer et al., 2018), and are distinguished by a unique combination of morphological features absent in other Myrmicinae (Bolton, 1982). Importantly, *Melissotarsus* is much more specialized, including a ventrally enlarged head and aberrantly modified legs that make it impossible for workers to walk outside their network of tunnels (Khalife et al., 2018).

Using scanning microscopy, Fisher and Robertson (1999) described groove-shaped openings along the buccal margin of *Melissotarsus beccarii* (= *M. emeryi*) workers, with silk-like threads oozing out. Hölldobler et al. (2014) confirmed with histology a large hypostomal silk gland situated in the anterior portion of the head of workers in three *Melissotarsus* species. Scanning micrographs showed similar buccal grooves in workers of *Rhopalomastix* sp. also, although histological examination did not reveal any gland cells (Hölldobler et al., 2014). Another exocrine peculiarity of *Melissotarsus*, not found in any other ants, is the occurrence of a gland in the expanded basitarsus of all six legs of workers as well as queens (Delage-Darchen, 1972). This finding in *M. weissi* (= *M. titubans*) was based on high magnification dissection microscope examinations of a crater-shaped sieve plate on the outer distal portion of each basitarsus. Scanning microscopy of these sieve plates in *M. beccarii* and *M. weissi* revealed the presence of round pores with a diameter of 0.5 µm and toothpaste-like material oozing out (Billen, 2009). Histological confirmation for the presence of glands was provided by Hölldobler et al. (2014). Both the silk glands and the basitarsal glands correspond to class-3 exocrine glands (standard classification by Noirot and Quennedey, 1974). Such glands are made up of numerous bicellular units with a secretory cell and a duct cell. A cuticular end apparatus functions as a draining device to transport secretions from the secretory cell into the duct cell. The cuticular canal in the duct cells typically has a diameter of 0.5-1 µm and opens to the outside through a pore of the same size.

We compared exocrine glands in these two genera showing similar lifestyles but different degrees of morphological specialisation. We discuss the function of head and leg glands relative to caste-specific tasks, including colony founding.

2. Material and methods

Rhopalomastix sp. was collected throughout Singapore, with a range of host trees as well as partner diaspidid genera (details in Yong et al., 2019). *Rhopalomastix janeti* was collected in Cha-Om, Khaeng Khoi district, Saraburi province, Thailand. *Melissotarsus beccarii* was sampled in the Western Cape province of South Africa (May 2017 and June 2018; Nardouw farm near Clanwilliam (32°0'4.26"S, 18°50'20.40"E)). Colony fragments from several host trees (*Leucospermum praemosum* in family Proteaceae) were collected by sawing off inhabited branches. These were brought back to the laboratory where extremities were kept moist with wet tissues, and both diaspidids (*Morganella conspicua*) and ants remained alive for about 6 weeks. For observations of silk spinning behaviour, the bark was scraped under a compound microscope, allowing photos and videos.

To allow proper penetration of the various chemicals used during tissue processing, ants were cut prior to fixation to create a sufficiently large opening. The anterior part of the heads was transversely cut off behind the compound eyes. In order not to lose the legs of the very small ants during tissue processing, they were left attached to the thorax. The distal tarsomeres were cut off so that the tissue processing chemicals could penetrate the basitarsus. Only at the moment of embedding, the basitarsi of the fore-, mid- and hindlegs were separated and individually embedded and sectioned. Tissue processing started with fixation in 2% cold glutaraldehyde in a 0.05 M Na-cacodylate buffer with 0.15 M saccharose and postfixation in 2% osmium tetroxide postfixation in the same buffer. After dehydration in a graded acetone series and embedding in Araldite, serial semithin sections with a thickness of 1 µm were made with a Leica EM UC6 ultramicrotome, and after staining with methylene blue and thionin viewed in an Olympus BX-51 microscope. Histological data are based on the study of 22 *Melissotarsus* and 15 *Rhopalomastix* workers. Calculation of cell numbers for the silk gland was achieved by counting the cumulative number of secretory cells on serial sections with an interval that corresponds with average cell diameter; for the basitarsal glands the number can be inferred from the number of pores that are externally visible (see further). Thin sections of 70 nm were double stained with lead citrate and uranyl acetate and examined in a Zeiss EM900 electron microscope. Material for scanning microscopy (including roof material) was gold-sputtered and examined in a JEOL JSM-6360 scanning microscope. Some heads for SEM were treated with 10% KOH to dissolve the soft tissues in order to look at the cuticular parts. The material used for scanning microscopy comprised 33 workers, 4 queens and 2 males for *Melissotarsus*, and 22 workers for *Rhopalomastix*.

3. Results

3.1. Silk glands

Along its entire length, the exterior side of the semicircular margin of the buccal cavity in *Melissotarsus* workers shows a linear series of perpendicular grooves with a length of 5 µm and a

width of 0.2-0.4 μm (Fig. 1A). The total number of grooves is around 50, with a regular spacing of 7-8 μm , and thread-like material (diameter of 0.6-1 μm) oozing out of the anterior side of the grooves (Fig. 1B). Similar grooves also exist in queens (Fig. 1E), but not in males (Fig. 1F). *Rhopalomastix* workers also display a series of 40-50 grooves of comparable size along the margin of their buccal cavity (Fig. 1C), with similar thread-like material oozing out (Fig. 1D). The grooves are not arranged linearly as in *Melissotarsus*, but occur on top of irregular elongated knob-like elevations along the buccal cavity margin (Fig. 1D).

Histological semithin sections through the head of both *Melissotarsus* and *Rhopalomastix* workers revealed a large silk gland that consists of many secretory cells and duct cells (Fig. 2). In both genera, this gland corresponds to class-3 and occurs in the anterior ventral portion of the head as well as inside the proximal part of the mandibles (Fig. 2A). In *Melissotarsus*, we counted 176 ± 26 secretory cells with a diameter of $45.2 \pm 3.9 \mu\text{m}$ ($N = 6$); variability in cell number is likely related to variability in body size, but we did not investigate this. These cells are loaded with abundant round and dark-staining secretory vesicles that push the round nucleus to a peripheral position (Fig. 2C). The ducts from all secretory cells converge to the buccal margin, where they open through the grooves. The transition of the round ducts to the slit-like grooves occurs through the intricate spongy cuticle of the buccal margin (Fig. 2B). In *Rhopalomastix*, around 50 secretory cells with an average diameter of $37.8 \pm 4.9 \mu\text{m}$ ($N = 6$) were found ventrally inside the head capsule, and an additional dozen cells inside each mandible. The nuclei are peripherally located because the main part of the cells is occupied by a large homogeneously staining space, that represents the end apparatus (Fig. 2D). The ducts of the secretory cells located inside the mandibles open directly through the mandibular cuticle (Fig. 2D, left inset), whereas ducts from the secretory cells in the head capsule open through the buccal margin grooves. *Rhopalomastix* does not have a spongy cuticle in its buccal margin, instead ducts retain their straight round outline when crossing the cuticle (Fig. 2D, right inset).

Ultrastructural observation of the secretory cells in *Melissotarsus* showed the end apparatus amidst densely packed dark secretory vesicles (Fig. 3A), and this makes the end apparatus difficult to recognize on light microscopy sections. Unexpectedly, this end apparatus is branched (Fig. 3B), as can also be seen in KOH-treated material in which soft tissues were dissolved and only cuticular material remained (Fig. 3C). The major cytoplasmic element besides the secretory vesicles and the end apparatus are stacks of rough endoplasmic reticulum (Fig. 3D). In *Rhopalomastix*, silk gland cells have a straight end apparatus that is surrounded by a large space filled with secretions (there are no vesicles) (Fig. 3E,F). The remaining part of the cytoplasm is rough endoplasmic reticulum (Fig. 3G).

3.2. Basitarsal glands

Underlying their tunnelling behaviour in healthy trees, *Melissotarsus* workers are characterized by the aberrant morphology of mid- and hindlegs, such as highly expanded coxae and basitarsi

(Khalife et al., 2018). For the forelegs, the ventral region of tarsomeres is equipped with dense hairs (Fig. 4A), whereas the mid- and hindlegs have few hairs only, and a semicircular ring of stout basitarsal pegs (Fig. 4B). The enlarged basitarsi of the three leg pairs correspond to the presence of a class-3 exocrine gland, with ducts opening through a peculiar sieve plate on the outer distal part (Fig. 4A-F). As Bernadette Delage-Darchen (1972) was the first to recognize this gland opening, we designate the basitarsal gland in *Melissotarsus* as the “Delage-Darchen gland”. The sieve plates are surrounded by a round crater-like rim, and contain small pores with a diameter of 0.3 μm , from which solid threads with a similar diameter ooze out. Both the diameter of the sieve plate crater and the number of pores, and hence also the number of gland cells, is highest in the forelegs (where the basitarsus is most enlarged) and lowest in the midlegs (Figs 4C-E; Table 1). Queens have a similar Delage-Darchen gland. Histological sections show duct cells leading from the sieve plate to the interior of the basitarsus where they connect with large secretory cells that have a diameter of up to 50 μm . These cells are almost completely filled with large spherical and pale vacuoles that reach a diameter of 15 μm (Fig. 4F). Winding through each cell is the end apparatus, that can be branched as in the silk gland (Fig. 4F). The remaining cytoplasm is restricted to small darker staining areas in between the vacuoles, and contains well-developed stacks of rough endoplasmic reticulum and Golgi bodies (Fig. 4G). Enlarged basitarsi and Delage-Darchen glands are lacking in *Rhopalomastix*, although some scattered basitarsal class-3 gland cells are present with separated duct openings (Fig. 4H).

Besides the Delage-Darchen gland, the fore-basitarsus of *Melissotarsus* also contains an epithelial class-1 gland (Fig. 5A), that is equally found in *Rhopalomastix* (Fig. 5B). This gland was described for several ant species as the antenna-cleaner gland (Schönitzer et al., 1996). In both *Melissotarsus* and *Rhopalomastix*, this gland is located along a longitudinal comb-like groove in the ventral proximal part of the basitarsus and made up of tall columnar cells with a thickness of 25-30 μm . Externally, the cuticle of this groove appears as a series of micro-spines of 2-3 μm long, that is separated from the epithelial cells by a subcuticular space (Fig. 5C), originally described as ‘bande poreuse’ (porous strip) by Janet (1894). The gland cells display an extensive labyrinth of basal invaginations (Fig. 5D) and apical microvilli (Fig. 5E), numerous mitochondria and smooth endoplasmic reticulum (Fig. 5E).

3.3. Silk production

Whereas the tarsomeres of mid- and hindlegs are almost smooth (compare Figs 4A and 4B), the foreleg tarsomeres of both *Melissotarsus* and *Rhopalomastix* are very hairy (Fig. 6). This corresponds to the involvement of forelegs in silk spinning, as observed when breaches in the roof of tunnels were repaired by workers (Fisher and Robertson, 1999; N. Cyrille and C. Peeters, unpublished). The resulting construction (Fig. 7 A-B) is a mesh of silk threads and wood pieces. Strips of older roof were easily cut away from branches, revealing a coherent and rigid structure

179 (Fig. 7 C-D). SEM comparison of freshly repaired tunnel roofs vs. strips of older roof hint that silk is
180 bonded with wood in the latter (Fig. 7 E-F).

181 4. Discussion

182 4.1. Silk glands

183 The silk glands in *Melissotarsus* adults (Fisher and Robertson, 1999; Hölldobler et al., 2014)
184 are unique among aculeate Hymenoptera. Here we confirm and document this extraordinary silk
185 gland in queens and workers, and we also show its existence in the sister genus *Rhopalomastix*.
186 The silk gland is not homologous to other known head glands, and we consider it to have evolved
187 *de novo*. Rough endoplasmic reticulum is the dominant component of secretory cells in both
188 *Melissotarsus* and *Rhopalomastix*, in agreement with the elaboration of silk proteins. However,
189 remarkable ultrastructural differences between genera include numerous secretory vesicles and a
190 branched end apparatus in *Melissotarsus*, whereas the secretion is collected in one large space
191 around a straight end apparatus in *Rhopalomastix* (Fig. 3). A branched end apparatus (connecting
192 secretory and duct cells) is highly unusual for class-3 glands in ants, although it is a common
193 feature in wasps (Delfino et al. 1979, 1983). This branched end apparatus in *Melissotarsus*
194 amounts to an important divergence from *Rhopalomastix*, apparently related to the higher
195 secretory activity of individual secretory cells in *Melissotarsus*. Another striking difference is a more
196 than two-fold increase in the number of secretory cells in *Melissotarsus*, and these are also larger
197 than in *Rhopalomastix*. In *Melissotarsus*, all the ducts (including those from secretory cells inside
198 the mandibles) release silk through the narrow grooves along the buccal margin, whereas in
199 *Rhopalomastix* secretory cells inside the mandibles open directly through the mandibular cuticle
200 and only secretory cells in the head capsule open through the buccal grooves. The total number of
201 grooves is around 50 in both genera despite connecting to substantially different numbers of
202 secretory cells. In *Rhopalomastix*, the number of secretory cells in the head capsule more or less
203 equals the number of grooves, this 1:1 ratio means that each duct cell connects to a groove.
204 *Melissotarsus*, in contrast, has considerably more secretory cells than grooves, a ratio around 3:1,
205 and this results from ducts merging as they pass through the unusual spongy cuticle of the buccal
206 margin (Fig. 2B). The above comparison suggests that *Melissotarsus* has an increased ability to
207 secrete silk in spite of the number of grooves being constrained by the perimeter of the buccal
208 margin. The conspicuous ventral expansion of the head capsule of *Melissotarsus* workers allows to
209 accommodate atypically large opener muscles for the mandibles (Khalife et al., 2018), but also a
210 larger silk gland. This evolutionary divergence from *Rhopalomastix* has crucial ecological
211 consequences (see below).

212 4.2. Basitarsal glands

213 Highly modified coxae and basitarsi of mid- and hindlegs in *Melissotarsus* indicate a total
214 commitment to life inside tunnels, resulting in workers being unable to walk and forage outside
215 (Khalife et al., 2018). In contrast, *Rhopalomastix* lack such specialized legs and can walk on the
216 outside of host trees (Yong et al., 2019). The existence of the Delage-Darchen gland in the
217 basitarsus of all legs in *Melissotarsus* is a further difference between these sister genera. The size
218 of the sieve plates and the number of duct openings, hence the number of secretory cells, is
219 considerably higher in the forelegs (Fig. 4 C-E). We lack direct evidence about the function of the
220 Delage-Darchen gland, although the ultrastructural characteristics of a well-developed rough
221 endoplasmic reticulum and solid material oozing out of the pores indicate a proteinaceous
222 secretion rather than volatile substances such as hydrocarbons with pheromonal function.

223 The few isolated class-3 cells found in the basitarsus of *Rhopalomastix* are not homologous to
224 the Delage-Darchen gland, but instead resemble the tarsomere glands (see number 16 in Billen,
225 2009). Both genera also have an epithelial 'antenna cleaner gland' in the proximal part of the
226 foreleg basitarsi, although this is a fairly common exocrine structure among ants (Schönitzer et al.,
227 1996) and Hymenoptera in general (Basibuyuk and Quicke, 1994).

228 4.3. Silk production

229 *Melissotarsus* and *Rhopalomastix* belong to the large subfamily Myrmicinae where cocoons
230 have been lost and pupae are always naked. The exceptional existence of silk glands in the female
231 adults of two sister genera is linked with their tunnelling activity under the bark of healthy trees. Silk
232 is used to anchor wood particles (frass) in the roofs of tunnels. With the increase in diameter of
233 growing host branches, existing tunnels can fissure and need continuous upkeep. Moreover,
234 tunnels must be immediately repaired following an intrusion, hence silk is a crucial component of
235 nest defence (especially in *Melissotarsus* since the almost blind and minute workers have lost the
236 sting and walk awkwardly outside tunnels). Silk is not directly involved in interactions between ants
237 and diaspidid scale insects, but it guarantees the latter's safety and certainly contributed to the
238 evolutionary success of this mutualism.

239 Spinning behaviour is elaborate in *Melissotarsus* and involves conspicuous adaptations of the
240 distal forelegs (Fisher and Robertson, 1999). The hairy tarsomeres on the forelegs function to draw
241 out the silk filaments, that are then applied to the perimeter of a breach. In *Rhopalomastix*, this
242 process is somewhat distinct and less understood (Yong et al., 2019). Another significant
243 difference between these two genera is the Delage-Darchen gland and its proteinaceous secretion
244 (indicated by the rough endoplasmic reticulum, Fig. 4G). Tunnel roofs are not rigid immediately
245 after repairs, but a few days later they resist better if physical pressure is applied from the outside
246 (N. Cyrille and C. Peeters, unpublished). Moreover, the inner surface of roofs looks much
247 smoother and compacted than the outer (Fig. 7C,D). We suspect that the secretion of the Delage-
248 Darchen glands is added to the silk&wood mesh of tunnel roofs to increase their stiffness. This

249 would be analogous to the chemical process of curing that hardens any polymers by forming
250 covalent bonds. When polymer chains are crosslinked, the material becomes more rigid. Our
251 hypothesis that the Delage-Darchen secretions bond the wood and silk together is supported by
252 the spatial location of the sieve plates, making direct contact of foreleg tarsomeres with tunnel
253 roofs possible. The presence of a functional Delage-Darchen gland in mid- and hindlegs is less
254 intuitive, but their basitarsi may also apply secretions to tunnel roofs.

255 In contrast, *Rhopalomastix* lack enlarged basitarsi and Delage-Darchen glands. The roofs of its
256 tunnels are more of an aggregate of silk and wood that is not firm and compressed as those of
257 *Melissotarsus*, and tunnels may be more susceptible to intrusion by other ants and insects.
258 Noteworthy is the contrast in behaviours after we breached tunnels: *Melissotarsus* workers
259 immediately start to spin silk and repair (Peeters et al., 2017), while *Rhopalomastix* escape to the
260 safety of adjacent tunnels (Yong et al., 2019).

261 Silk is a material with valuable properties (Sutherland et al., 2010) that can serve a variety of
262 purposes across insects. In aculeate Hymenoptera, cocoons are spun by last instar larvae with silk
263 secreted in their labial gland. This silk has been repurposed as a building material in arboreal ants
264 from three genera in subfamily Formicinae (Hölldobler and Wilson, 1990). Silk fibers entangled
265 together form strong and compliant sheets (Crewe and Thompson, 1979), with sticky properties
266 appropriate to bind both living leaves and wood particles. Most famously, *Oecophylla* workers use
267 chains to pull living leaves closer, then weave these together using larvae as living shuttles
268 (Wheeler, 1915 p. 327-329). In *Camponotus*, species from three subgenera (Asia and South
269 America) also use larvae to weave their nests, but they incorporate sand particles, detritus and bits
270 of plants into the silk sheets (e.g. Maschwitz et al., 1985). In *Polyrhachis*, workers of a few
271 subgenera use larvae to weave silk pavilions on the undersides of leaves, or mix silk with
272 vegetable fragments to construct hard walls or close off a folded leaf; a few species steal spider
273 silk instead (Dorow and Maschwitz, 1990; Robson and Kohout, 2007). In *P. (Cyrtomyrma) delecta*,
274 'girder' structures of silk (> 1cm long) can give extra strength to leaf nests (Tranter and Hughes,
275 2015). A different use of silk from ant larvae is found in *Harpegnathos saltator* (Ponerinae), where
276 underground nest chambers are lined with empty cocoons, presumably to control humidity
277 (Peeters et al., 1994). But 60% of extant ants (C. Peeters, unpublished) have eliminated cocoons.
278 In subfamilies Myrmicinae and Dolichoderinae, many arboreal species construct their nests outside
279 trees using a rigid carton material, composed of masticated and moistened plant fibres; fungi can
280 be involved in some species (Rohe and Maschwitz, 2003; Weissflog et al., 2017). Only in
281 *Melissotarsus* and *Rhopalomastix* can adults use silk for nest construction, but the structural
282 advantages relative to carton are yet unclear. However, the instant availability of silk that is mixed
283 with wood chips lying around inside tunnels allows emergency repairs that can be completed in
284 less than 15 mins (N. Cyrille & C. Peeters, unpublished), a vital defence against potential invaders.

285

286 4.4. Evolutionary divergence between *Melissotarsus* and *Rhopalomastix*

287 Based on external morphology, Bolton (1982) concluded that these sister genera represent
288 two stages on a single adaptive line, with *Rhopalomastix* being more generalized. The greatly
289 enlarged heads in both genera correspond to powerful mandible muscles (Khalife et al., 2018), and
290 both stand out among ants by the ability of adults to produce silk. However, extreme adaptations in
291 mid- and hindlegs (Khalife et al., 2018) are restricted to *Melissotarsus*. Distal leg structures in
292 Hymenoptera can be highly modified, linked to efficient walking and cleaning the body surface
293 (Beutel et al., 2020). *Melissotarsus* legs are a novel illustration of this evolutionary versatility, with
294 enlarged basitarsi and aberrant tarsomeres (hairy in forelegs; highly reduced in mid- and hindlegs).
295 Our results on the silk gland and Delage-Darchen gland add to this evolutionary divergence. The
296 silk gland is considerably larger in *Melissotarsus*, with more than double the secretory cells present
297 in *Rhopalomastix*. Moreover, ultrastructure reveals modified organelles in *Melissotarsus*
298 suggesting augmented silk synthesis in each secretory cell. Given the key role of silk for defence in
299 these two genera, the ability to produce larger quantities of silk provides additional benefits for
300 *Melissotarsus*. This is combined with exocrine glands inside basitarsi implicated in hardening the
301 silk&wood roofs. More secure tunnels in *Melissotarsus* likely account for loss of the sting in
302 workers, unlike in *Rhopalomastix*.

303 Caste polyphenism in ants allows queens and workers to diverge in morphology as adaptation
304 for different activities. Young queens in *Melissotarsus* and *Rhopalomastix* need to mate, fly in
305 search of a host tree, walk on its trunk before they chew a shelter under bark. *Melissotarsus*
306 queens show similar leg modifications as workers, but their midlegs do not face upwards and they
307 can walk normally. During colony founding, silk secretion is likely essential to secure an initial
308 tunnel. A dealate infertile queen in an established colony of *Melissotarsus* was observed repairing
309 a breach in tunnels (N. Cyrille and C. Peeters, unpublished), and exhibiting the same spinning
310 behaviour as workers'. An expanded use of silk in *Melissotarsus* constructions allows for better
311 protection against arboreal ants and other predators, compared to *Rhopalomastix*.

312 5. Acknowledgements

313 We thank Gordon Yong (Singapore) and Weeyawat Jaitrong (Bangkok) for help in collecting
314 *Rhopalomastix*, Dr Pitchaya Pothinuch for hospitality on his mango plantation, and Bennie
315 Bezuidenhout (Nardouw farm) for his enthusiastic support with *Melissotarsus* fieldwork. Our
316 sincere thanks also go to An Vandoren for her assistance in sectioning and to Alex Vrijdaghs for
317 his help in scanning microscopy. Nathan Cyrille helped with images of the tunnel roof. We are
318 grateful to Robin Crewe for useful discussions about silk and polymer chemistry, to Roberto Keller
319 for general guidance with morphology, and to two anonymous reviewers for constructive
320 comments on the manuscript.

321 6. References

- 322 Basibuyuk, H.H. and Quicke, D.L.J., 1994. Evolution of antennal cleaner structure in the
323 Hymenoptera (Insecta). Norw. J. Agric. Sci., Suppl. 16, 199-206.
- 324 Beutel, R.G., Richter, A., Keller, R.A., Hita Garcia, F., Matsumura, Y., Economo, E.P. and Gorb,
325 S.N., 2020. Distal leg structures of the Aculeata (Hymenoptera): A comparative evolutionary
326 study of *Sceliphron* (Sphecidae) and *Formica* (Formicidae). J. Morphol. DOI:
327 10.1002/jmor.21133
- 328 Billen, J., 2009. Occurrence and structural organization of the exocrine glands in the legs of ants.
329 Arthropod Struct. Dev. 38, 2-15.
- 330 Billen, J. and Šobotník, J., 2015. Insect exocrine glands. Arthropod Struct. Dev. 44, 399-400.
- 331 Bolton, B., 1982. Afrotropical species of the myrmicine ant genera *Cardiocondyla*, *Leptothorax*,
332 *Melissotarsus*, *Messor* and *Cataulacus* (Formicidae). Bull. Brit. Mus. (Nat. Hist.) (Entomol.) 45,
333 307-370.
- 334 Blaimer, B.B., Ward, P.S., Schultz, T.R., Fisher, B.L. and Brady, S.G., 2018. Paleotropical
335 diversification dominates the evolution of the hyperdiverse ant tribe Crematogastrini
336 (Hymenoptera: Formicidae). Insect Syst. Div. 2: 3, 1-14 (doi: 10.1093/isd/ixy013)
- 337 Crewe, R.M. and Thompson, P.R., 1979. *Oecophylla* silk: functional adaptation in a polymer.
338 Naturwissenschaften 66, 57-58.
- 339 Currie, C.R., Poulsen, M., Mendenhall, J., Boomsma, J.J. and Billen, J., 2006. Coevolved crypts
340 and exocrine glands support mutualistic bacteria in fungus-growing ants. Science 311, 81-83.
- 341 Delage-Darchen, B., 1972. Une fourmi de Côte-d'Ivoire: *Melissotarsus titubans* Del., n. sp. Insect.
342 Soc. 19, 213-226.
- 343 Delfino, G., Marino Piccioli, M.T. and Calloni, C., 1979. Fine structure of the glands of Van der
344 Vecht's organ in *Polistes gallicus* (L.) (Hymenoptera Vespidae). Monitore zool. Ital. (N.S.) 13,
345 221-247.
- 346 Delfino, G., Marino Piccioli, M.T. and Calloni, C., 1983. Ultrastructure of the venom glands in *Polistes*
347 *gallicus* (L.) (Hymenoptera Vespidae). Monitore zool. Ital. (N.S.) 17, 263-277.
- 348 Dorow, W.H.O. and Maschwitz, U., 1990. The *arachne*-group of *Polyrhachis* (Formicidae:
349 Formicinae): weaver ants cultivating Homoptera on bamboo. Insectes Soc. 37, 73-89.
- 350 Fisher, B.L. and Robertson, H.G., 1999. Silk production by adult workers of the ant *Melissotarsus*
351 *emeryi* (Hymenoptera, Formicidae) in South African fynbos. Insect. Soc. 46, 78-83.
- 352 Grasso, D.A., Mori, A., Le Moli, F. and Billen, J., 2005. Morpho-functional comparison of Dufour's
353 gland in the female castes of the Amazon ant *Polyergus rufescens* (Hymenoptera,
354 Formicidae). Zoomorphology 124, 149-153.
- 355 Hölldobler, B. and Wilson, E.O., 1990. The Ants. Harvard UP
- 356 Hölldobler, B., Obermayer, M., Plowes, N.J.R. and Fisher, B.L., 2014. New exocrine glands in
357 ants: the hypostomal gland and basitarsal gland in the genus *Melissotarsus* (Hymenoptera:
358 Formicidae). Naturwissenschaften 101, 527-532.
- 359 Janet, C., 1894. Sur l'organe de nettoyage tibio-tarsien de *Myrmica rubra* L. race *laevinodis* Nyl.
360 Annls Soc. Entomol. France 63, 691-704.
- 361 Khalife, A., Keller, R., Billen J., Hita Garcia, F., Economo, E. and Peeters, C., 2018.
362 Skeletomuscular adaptations of head and legs of *Melissotarsus* ants for tunnelling through
363 living wood. Front. Zool. 15, 30.
- 364 Maschwitz, U., Dumpert, K. and Schmidt, G., 1985. Silk pavilions of two *Camponotus* (*Karavaievia*)
365 species from Malaysia: description of a new nesting type in ants (Formicidae: Formicinae). Z.
366 Tierpsychol. 69, 237-249.

367 Noirot, C. and Quennedey, A., 1974. Fine structure of insect epidermal glands. Annu. Rev.
368 Entomol. 19, 61-80.

369 Peeters, C., Foldi, I., Matile-Ferrero, D. and Fisher, B.L., 2017. A mutualism without honeydew:
370 what benefits for *Melissotarsus emeryi* ants and armored scale insects (Diaspididae)? PeerJ 5,
371 e3599.

372 Peeters, C., Hölldobler, B., Moffett, M. and Musthak Ali, T., 1994. "Wall-papering" and elaborate
373 nest architecture in the ponerine ant *Harpegnathos saltator*. Insectes Soc. 41, 211-218.

374 Robson, S. and Kohout, R., 2007. A review of nesting habits and socioecology of the ant genus
375 *Polyrhachis* Smith. Asian Myrmecol. 1, 81-99.

376 Rohe, W. and Maschwitz U., 2003. Carton nest building and trophobiont manipulation in the south-
377 east Asian ant *Dolichoderus sulcaticeps* (Mayr 1870) (Hymenoptera: Formicidae). J. Nat. Hist.
378 37, 2835-2848.

379 Schönitzer, K., Dott, H. and Melzer, R.R., 1996. The antenna cleaner gland in *Messor rufitarsis*
380 (Hymenoptera, Formicidae). Tissue & Cell 28, 107-113.

381 Sutherland, T.D., Young, J.H., Weisman, S., Hayashi, C.Y. and Merritt, D.J., 2010. Insect silk: one
382 name, many materials. Annu. Rev. Entomol. 55, 171-188.

383 Tranter, C. and Hughes, W.O.H., 2015. A preliminary study of nest structure and composition of
384 the weaver ant *Polyrhachis (Cyrtomyrma) delecta* (Hymenoptera: Formicidae). J. Nat. Hist. doi:
385 10.1080/00222933.2015.1103912

386 Weissflog, A., Kaufmann, E. and Maschwitz, U., 2017. Ant gardens of *Camponotus (Myrmotarsus)*
387 *irritabilis* (Hymenoptera: Formicidae: Formicinae) and *Hoya elliptica* (Apocynaceae) in
388 Southeast Asia. Asian Myrmecol. 9, e009001.

389 Wheeler, W.M. 1915. On the presence and absence of cocoons among ants, the nest-spinning
390 habits of the larvae and the significance of black cocoons among certain Australian
391 species. Annals Entomol. Soc. Am. 8, 323-342.

392 Yong, G., Matile-Ferrero, D. and Peeters, C., 2019. *Rhopalomastix* is only the second ant genus
393 known to live with diaspidid scale insects. Insect. Soc. 66, 273-282.
394

395 Table 1. Size and number of pores of the Delage-Darchen glands in the basitarsi of *Melissotarsus*
396 workers.

397

	sieve plate diameter (µm)	number of pores
foreleg	28.9 ± 2.4 (n = 11)	35.1 ± 3.1 (n = 8)
midleg	15.8 ± 0.4 (n = 5)	8.3 ± 1.0 (n = 4)
hindleg	22.4 ± 2.5 (n = 7)	19.8 ± 1.9 (n = 4)

398

Fig. 1. Ventral view scanning micrographs of the buccal cavity margin (BCM). **A.** *M. beccarii* worker. **B.** Detail of groove-shaped gland openings (arrows) along BCM in *M. beccarii* worker. Arrowheads indicate silk filaments. **C.** *Rhopalomastix* sp. worker. **D.** Detail of silk filament (arrowhead) coming out of groove (arrow). **E.** BCM with silk gland openings (arrows) in *M. beccarii* queen. **F.** BCM lacking silk gland openings in *M. beccarii* male. Md: mandibles.

Fig. 2. **A.** Semithin frontal section through head of *M. beccarii* worker showing numerous secretory cells of the silk gland (SG) in anterior portion of head and inside mandibles. **B.** SEM of cuticle of buccal cavity margin in *M. beccarii* worker after KOH treatment and cutting with razorblade along the angle shown by the white double arrow. Black double arrow indicates the extent of sponge-like cuticle (SC), through which silk gland ducts (SGd) cross before opening on buccal

margin as parallel grooves (white arrows). **C,D.** Semithin transverse sections through anterior head region with silk glands of *M. beccarii* worker (**C**) and *Rhopalomastix* sp. worker (**D**). Insets in D show for *R. sp.* how silk glands inside mandible open directly through mandibular cuticle (arrowheads left inset) and how silk gland ducts in head capsule retain their tube-shape when crossing through buccal margin (arrowhead in right inset). ant: antenna, br: brain, CE: compound eye, Md: mandibles, MM: mandibular muscles, MP: mouthparts, prG: prepharyngeal gland.

Fig. 3. Electron micrographs of the silk gland in workers of *M. beccarii* (A-D) and *R. sp.* (E-G). **A.** General view of secretory cell with numerous vacuoles (SV), together with duct cell (DC). **B.** Detail of branched end apparatus (EA) and surrounding secretory vacuoles (SV). Arrowheads

423
424
425
426
427
428
429

indicate connection between secretory cell and duct cell. **C.** SEM showing cuticular part of branched end apparatus after KOH-treatment. **D.** Detail of cytoplasm with rough endoplasmic reticulum (RER) and secretory vacuoles. **E.** General view of one secretory cell and duct cell (DC). **F.** Detail of straight end apparatus (EA), with arrowheads showing the discontinuous epicuticle that is a standard feature of class-3 gland cells. **G.** Detail of cytoplasm with rough endoplasmic reticulum. mv: microvilli, Nd: nucleus of duct cell, NF: nerve fibre, Ns: nucleus of secretory cell.

430

431 Fig. 4. Delage-Darchen glands in basitarsi of *M. beccarii* worker: **A.** Ventrolateral SEM of the
432 extremely hairy foreleg tarsomeres, showing Delage-Darchen gland sieve plate (DD). bt:
433 basitarsus, pc: pretarsal claws, ts: tibial spur, **B.** Ventrolateral view of mostly smooth midleg
434 tarsomeres, showing semicircular ring of basitarsal pegs (bp). **C-E.** Details of Delage-Darchen
435 gland sieve plate in basitarsus of foreleg (C), midleg (D) and hindleg (E); note toothpaste-like
436 secretion oozing out of pores. **F.** Semithin cross section through distal part of hindleg
437 basitarsus showing sieve plate (DD), duct cells (DC) and two highly vacuolar secretory cells
438 (SC) of Delage-Darchen gland. Note branching in the end apparatus (EA) at bottom. **G.**
439 Electron micrographs of Delage-Darchen gland secretory cells in midleg. **H.** Semithin cross
440 section through midleg basitarsus of *R. janeti* worker with class-3 tarsomere gland cells (TG).
441 ga: Golgi apparatus, RER: rough endoplasmic reticulum.

Fig. 5. Antenna-cleaner gland in foreleg basitarsus. **A.** Transverse section from *M. beccarii* worker with antenna-cleaner gland (ACG) and vacuolated cells of Delage-Darchen gland (DD). EA: end apparatus, sg: spiny groove. **B.** Transverse section from *R. janeti* worker. bc: part of basitarsal comb. **C-E.** Electron micrographs of secretory epithelium in *M. beccarii* worker: **C.** spiny groove in cuticle (sg), subcuticular space (ss) and apical cytoplasm. **D.** Basal invaginations (bi) and nerve fiber (nf) in basal cell part. **E.** Apical region with microvilli (mv) and smooth endoplasmic reticulum (SER). M: mitochondria, N: nucleus.

Fig. 6. Scanning micrographs of foreleg basitarsus in *M. beccarii* (A,C) and *R. janeti* (B,D), showing more hairy ventral tarsomeres in *M. beccarii*. ar: arolium, bt: basitarsus (= tarsomere t1), DD: sieve plate of Delage-Darchen gland, pc: pretarsal claws, t2-t5: tarsomeres 2-5.

Fig. 7. Silk constructions in *M. beccarii* inhabiting *Leucospermum praemorsum* trees. **A.** Mesh of silk threads that will anchor wood fragments (frass) during the repair of tunnel roofs. **B.** Intact roof at right, and section being repaired at left. **C.** Inner surface of silk&wood roof removed from branch, showing the compaction and rigid shape. **D.** Zoom on bonded silk&wood mesh, suggestive of reinforcement by Delage-Darchen gland secretion. **E.** and **F.** SEMs of the mesh of silk threads and wood from a freshly repaired and old tunnel roof, respectively.