2 **The basitarsal sulcus gland, a novel exocrine structure in ants**

3 Johan Billen¹, Adam Khalife², Fuminori Ito³, Nguyen Duc Anh⁴ and Flavia A. Esteves⁵

4 ¹ *Zoological Institute, University of Leuven, Naamsestraat 59, box 2466, B-3000 Leuven,*
5 *Belgium. e-mail: joan.billen@kuleuven.be*

6 ² *Sorbonne Université, CNRS, Institut d'Écologie et des Sciences de l'Environnement, 75005*
7 *Paris, France.*

8 ³ *Laboratory of Entomology, Faculty of Agriculture, Kagawa University, Ikenobe, Miki 761-*
9 *0795, Japan*

10 ⁴ *Systematic Zoology Laboratory, Department of Biological Sciences, Tokyo Metropolitan*
11 *University, Tokyo 192-0397, Japan*

12 ⁵ *California Academy of Sciences, 55 Music Concourse Dr., San Francisco, CA 94941, U.S.A.*

13 **Keywords:** morphology; histology; ultrastructure; basitarsus; exocrine glands

14 **Running title:** the sulcus gland, a novel exocrine structure in ants

15 **Contact address:**

16 Johan Billen, KU Leuven, Zoological Institute, Naamsestraat 59, box 2466, B-3000 Leuven,
17 Belgium

18 Tel : (32) 16 323975

19 Fax : (32) 16 324575

20 E-mail: joan.billen@kuleuven.be

22 **Abstract**

23 The basitarsus of the mid- and/or hindlegs of several Amblyoponinae ants shows a deep
24 longitudinal groove or sulcus on its anterior face in workers and queens. Histological
25 examination reveals this sulcus is associated with a conspicuous novel epithelial gland, which
26 brings the number of exocrine glands in the legs of ants to 25. The ultrastructural
27 characteristics of the gland show the presence of a well-developed smooth endoplasmic
28 reticulum. This is indicative for the elaboration of a non-proteinaceous and thus possibly
29 pheromonal secretion. Behavioural observations show that this secretion is collected by the
30 tarsomeres and spread onto the brood and nest, suggesting a role in nestmate recognition. A
31 similar basitarsal sulcus gland was also found in *Nothomyrmecia*, *Paraponera* and
32 *Tetraponera*, which represents both a wide phylogenetic and ecological distribution, as it
33 includes arboreal, ground-dwelling as well as subterranean taxa.

34 **1. Introduction**

35 Since the illustrious Carl Linnaeus introduced the binomial scientific names for animals
36 and plants in the 18th century, hundreds of thousands of such 'Latin names' have been given
37 by taxonomists. The shortest animal species name is that of the greater evening bat *la io* in
38 Southeast Asia, the tongue-twisting longest is given to *Parastratiosphecomyia*
39 *stratiosphecomyioides*, a fly species in Thailand. The specific (second) part of the name often
40 refers to an anatomical character (e.g., the ant *Acromyrmex octospinosus* has eight
41 prominent spines on its thorax), reflects geographical distribution information (e.g., *Atta*
42 *texana*), or has been given as a tribute to a person (e.g., *Camponotus foreli*, called in honour
43 of the eminent Swiss myrmecologist Auguste Forel). An interesting example of anatomically
44 informative specific names is found in the amblyoponine ant genus *Stigmatomma*, in which
45 three species from Madagascar have been named according to the appearance of the
46 basitarsus in their mid- and hindlegs using wording from Malagasy language (Esteves and
47 Fisher, 2016): *Stigmatomma roahady* refers to the presence of a longitudinal sulcus or groove
48 on the anterior face of both the meso- and metabasitarsus (*roa* means 'two', *hady* means
49 'sulcus'), *S. irayhady* displays such sulcus only on the mesobasitarsus (*iray* means 'one'), and
50 *S. tsyhady* does not have any sulcus (*tsy* means 'absence').

The basitarsal sulcus is found in seven out of the eight *Stigmatomma* species occurring in the Malagasy region (Esteves and Fisher, 2016). A similar-shaped sulcus has been documented in the meso- and/or metabasitarsus of other Amblyoponinae (*Fulakora chilensis*, misidentified as *F. armigera*, *F. mystriops*, *Stigmatomma pallipes*, *S. pluto*, *Myopopone castanea*, and *Xymmer muticus*; Keller, 2011), as well as Myrmeciinae, Paraponerinae and Pseudomyrmecinae (Ward, 1990).

We here present a comparative study that checked for the presence of glandular tissue in the meso- and metabasitarsus of Amblyoponinae, Myrmeciinae, Paraponerinae, and Pseudomyrmecinae species, and describe a novel gland that is associated with the basitarsal sulcus. In addition, the availability of live *Myopopone castanea* allowed us to perform some behavioural observations related with their sulcus glands on the mid- and hindleg basitarsi.

2. Material and methods

A live queenright colony of *Myopopone castanea* was collected in Tay Yen Tu Nature Reserve (N21.17, E106.72), Bac Giang, Vietnam, in April 2017, and kept at 25°C in an artificial nest with plaster of Paris floor (for description, see Ito et al., 2020). Behavioural observations took place between April and June 2018, when the colony was queenless with approximately 80 workers. The ants were fed mealworms though workers mostly got their nutrients from larval hemolymph feeding (LHF: Ito, 2010). Eighteen workers were individualized with oil-based paint markers and observed between April 4th and 11th. Then, between April 11th and May 8th, 40 workers were followed for 10 minutes after they performed lateral leg-rubbing (LLR, see 3.3). Furthermore, 20 random workers were also followed for 10 minutes to compare behaviour with and without leg-rubbing. We tentatively coated the sulci in some workers with nail polish, with the expectation it would prevent secretion discharge and that the ants would in turn display some modified behaviour. Pictures and videos were taken using a handheld Canon EOS 550D (Tokyo, Japan) reflex camera equipped with a Canon EF 100mm f/2.8L USM macro lens.

Worker's basitarsi were cut off and fixed in 2.5% cold glutaraldehyde (in 0.1 M Na-cacodylate buffer at pH 7.2) for 12 hours. Postfixation was done in 2% cold osmium tetroxide for 1 hour, followed by dehydration in a graded acetone series and embedding in Araldite. Blocks were sectioned at a thickness of 1 µm with a diamond knife using a Leica EM UC6

ultramicrotome (Wetzlar, Germany) and stained with 1% methylene blue. Serial semithin sections were examined with an Olympus BX-51 microscope (Tokyo, Japan) connected to a camera. Thin sections of 70 nm were double-stained with lead citrate and uranyl acetate and studied under a Zeiss EM900 electron microscope (Oberkochen, Germany). Similarly prepared basitarsi for histological and/or ultrastructural examination were also available for workers of *Nothomyrmecia macrops* (from Poochera, Australia), *Onychomyrmex hedleyi* (from Ravenshoe, Australia), *Paraponera clavata* (from Belém, Brazil), *Stigmatomma roahady* (from Andrambovato, Madagascar) and *Tetraponera rufonigra* (from Bangkok, Thailand).

Mounted specimens of several other species were obtained from the collections of the California Academy of Sciences, San Francisco, USA (Flavia Esteves) and Kagawa University, Takamatsu, Japan (Fuminori Ito). Details of all ant material used in this study are provided in Supplementary Table S1. The presence of a basitarsal sulcus was evaluated under a stereoscope (Leica M205C, Leica Microsystems, Switzerland) and through electron microscopy. Samples for scanning microscopy were mounted on aluminium stubs, coated with gold-palladium, and viewed under a Hitachi SU3500 (Hitachi High-Technologies, Japan) and a JEOL JSM-6360 scanning microscope (Tokyo, Japan). Note that micrographs of specimens bearing "AntWeb" labels were originally produced by Keller (2011), and are available on AntWeb.org (AntWeb, 2020).

3. Results

As the initial inspiration for our paper was a taxonomic revision of the genus *Stigmatomma* in the Malagasy region (Esteves and Fisher, 2016), we first made a comparative study to check for the presence of a basitarsal sulcus across the genus *Stigmatomma*. Checking 23 species with worldwide distribution (see Supplementary Table S2) revealed that a sulcus is present on the midleg basitarsi in almost all species (Fig. 1). The sulcus is present in both workers and queens, but seems variable in males, where it may be long, very short or just absent. The sulcus appears as a straight longitudinal groove on the anterior face of the basitarsus that runs along the central portion (*S. roahady*), proximal portion (*S. tsyhady* and *S. liebe*) or almost the entire length of the mesobasitarsus (the majority of species). Only *S. roahady* has a similar sulcus also on the metabasitarsus (Fig. 1B). The only species without any basitarsal sulcus are *S. reclinatum*, *S. rothneyi*, and *S. tsyhady*. In *S. besucheti*, the sulcus is more curved (Fig. 1K), while *S. luyiae* (only known from

112 Taiwan) presents on the anterior face of the basitarsus of its midlegs a round concavity filled
113 with short rod-like cuticular projections (Fig. 1L).

114 Following Keller (2011), we extended our observations to species of other
115 Amblyoponinae, and confirmed the presence of a longitudinal sulcus on the mesobasitarsus
116 in females of *Myopopone* (which have such sulcus also on the metabasitarsus, Fig. 2A-C),
117 *Fulakora* and *Xymmer* (Fig. 2D-F; Supplementary Table S2). Contrary to Keller (2011)
118 though, we found that the mesobasitarsal sulcus is present in *Adetomyrma* workers and
119 queens (Fig 2D). The sulcus is absent in *Amblyopone*, *Mystrium*, *Prionopelta*, and
120 *Onychomyrmex*, as also observed by Keller (2011). *Onychomyrmex*, however, has a peculiar
121 oval concavity surrounding rod-like cuticular projections on the ventrodistal face of the
122 metabasitarsus (Fig. 2G, see also Hölldobler and Palmer, 1989a), and which appears similar
123 to what is seen in *Stigmatomma luyiae* (Fig. 1L). In some *Prionopelta* species, a very small
124 transverse sulcus of hardly 3 μm exists on the ventrodistal region of the hindleg of workers
125 and queens (Hölldobler et al., 1992; Ito and Billen, 1998).

126 The presence of a basitarsal sulcus is not only restricted to Amblyoponinae. We confirm a
127 similar sulcus on the anterior face of both the midleg and hindleg basitarsi in several
128 *Myrmecia* species and *Nothomyrmecia macrops* (Myrmeciinae, Fig. 3A,B), and *Paraponera*
129 *clavata* (Paraponerinae, Fig. 3C). In Pseudomyrmecinae, the sulcus is present on the meso-
130 and metabasitarsus of *Myrcidris epicharis* and in all species of *Tetraponera* we examined
131 save *T. attenuata*, which did not present the sulcus on the mesobasitarsus (Fig. 3D;
132 Supplementary Table S2).

133 3.1 Light microscopy

134 Histological sections of the basitarsus show the longitudinal sulcus as a deep groove with
135 a width of 5-10 μm , lined by an invagination of the thick basitarsal cuticular wall. The depth of
136 the groove varies according to the size of the ants, ranging from 5-10 μm in the smaller
137 *Stigmatomma* up to 50 μm in the giant *Paraponera*. Along its entire length, no matter whether
138 it occurs in the midleg or hindleg, the sulcus is internally lined by a conspicuous glandular
139 epithelium with tall cylindrical cells (Fig. 4A-F). The thickness of the epithelium depends on
140 the size of the ant and ranges between 15 and 50 μm , whereas the non-glandular epidermis
141 elsewhere in the basitarsus hardly reaches a thickness of 2-3 μm (Fig. 4A-F). The round
142 nucleus has a diameter around 5 μm and is basally located (Fig. 4A-F).

143 Sections through the oval metabasitarsal sulcus in *Onychomyrmex* (and also through the
144 region with the small transverse metabasitarsal sulcus in *Prionopelta*, see Hölldobler et al.,
145 1992; Ito and Billen, 1998) revealed the presence of glandular tissue, although this appears
146 clearly different from the characteristic epithelium as described above (Fig. 4G). Contrary to
147 the sulcus gland epithelium, the gland cells in *Onychomyrmex* contain numerous spherical
148 lipid droplets and display a capricious connection with the cuticle (Fig. 4G).

149 **3.2 Electron microscopy**

150 Ultrastructural examination confirms the glandular nature of the sulcus epithelium. The
151 thick cuticle overlaying the epithelium displays an irregular texture that forms transcuticular
152 channels for the passage of the secretory products (Fig. 5A,B). At the bottom of the sulcus,
153 minuscule depressions are seen at the apical side of the cuticle (Fig. 5A inset). The apical cell
154 membrane of the epithelial cells is differentiated into a clear microvillar border (Fig. 5A,B).
155 The cytoplasm is characterized by a well-developed smooth endoplasmic reticulum (Fig.
156 5C,D), although some local accumulations of rough endoplasmic reticulum can also be found
157 (Fig. 5C). Further obvious cytoplasmic organelles include Golgi apparatus and numerous
158 dark-staining mitochondria (Fig. 5C-E). The basal cell membrane shows irregular
159 invaginations, while a thin amorphous basement membrane of 50 nm separates the
160 epithelium from the underlying hemolymph space (Fig. 5E).

161 **3.3 Behavioural observations**

162 Our observations of a captive colony of *Myopopone castanea* indicate that secretions
163 from the meso- and metabasitarsi were spread on the tarsomeres and gaster tip through a
164 particular behaviour that we refer to as leg-rubbing (LR). This behaviour can sometimes be
165 observed in other ant species while they groom (e.g., genus *Carebara*; Khalife, pers. obs.). In
166 *M. castanea*, however, LR was observed exclusively in the nest and occurred so frequently
167 that all attempts to coat the sulcus with nail polish failed because it could not dry. We
168 observed two types of LR. In the lateral leg-rubbing (LLR), which was commonly observed
169 and could last from a few seconds to 7 minutes, tarsomeres of both forelegs rubbed the
170 basitarsus of ipsilateral mid- and hindlegs while the remaining legs acted as stands to support
171 the body (Fig. 6A). In the posterior leg-rubbing (PLR), which was observed less frequently
172 and only lasted a few seconds, both hindlegs were rubbed against each other underneath the
173 gaster while the worker stood on its four other legs (Fig. 6B). Slow-motion playback of LLR

174 and PLR recordings confirmed that the tarsomeres directly touched the sulcus area during
175 both types of LR (see Supplementary Videos 1 and 2).

176 We monitored the activity of workers for 10 minutes after performing lateral leg-rubbing
177 (LLR group) or during random 10-minute intervals (control group; see Supplementary Table
178 S3). In the control group, three workers out of 20 performed LLR, PLR or both and were thus
179 removed from our dataset. In the LLR group, workers performed LLR again in 60% of the
180 observations and PLR in 55%. Brood care was observed more often in the LLR group (50% of
181 the observations) compared to the control group (18%). Larval care was particularly
182 important: a single worker groomed and patted up to 14 larvae in a 10-minute interval.
183 Workers walked around in the nest less frequently in the LLR group than in the control group
184 (50% of the observations compared to 82%). Grooming of a nestmate worker was rarely
185 observed in both groups (15% for the LLR group, 12% for the control group). In the LLR
186 group, 13% of the workers went outside the nest compared to none in the control group.
187 Larval hemolymph feeding (LHF) never happened in any of our 60 observations, but co-
188 occurrence of LR and LHF was observed twice apart from these trials. In the first case, a
189 worker repeatedly performed consecutive LLR and self-grooming while feeding on the
190 hemolymph of a single larva. In the second case, another worker performed LLR, brood care
191 and LHF, then groomed a nestmate and repeated this sequence on another larva.

192 **4. Discussion**

193 The presence of an extraordinary diversity of exocrine glands in social insects, and in ants
194 in particular, is commonly known (Hölldobler and Wilson, 1990; Billen and Šobotník, 2015). In
195 their legs only, ants can have 20 different glands (Billen, 2009). This astonishing number has
196 increased to 24 with the description of a footsole gland in the hindleg distal tarsomeres of
197 *Protanilla wallacei* (Billen et al., 2013) and the recent discovery of 3 glands in the foreleg
198 coxae of *Discothyrea* ants (Billen et al., 2020). The present description of the novel basitarsal
199 sulcus gland therefore represents the 25th leg gland so far known in ants.

200 The structural characteristics of the basitarsal sulcus gland correspond with class-1
201 glands according to the standard classification by Noirot and Quennedey (1974), in which
202 epithelial secretory cells accumulate the precursor molecules from the underlying
203 hemolymph space and metabolize them into the final secretory products, which are eventually

204 discharged to the outside through the external cuticle. This transepithelial transport is
205 facilitated by obvious surface-increasing basal invaginations as well as apical microvilli. The
206 irregular texture and minuscule slits on the surface of the cuticle overlaying the glandular
207 epithelium (see Fig. 5 inset) provides a trajectory for the secretion to reach the outside.
208 Similar cuticular modifications for class-1 glands have also been reported for several other leg
209 glands in ants (Billen, 2009), for the mandibular pit gland in *Brachyponera sennaarensis*
210 (Billen and Al-Khalifa, 2016) and for the basimandibular gland in *Strumigenys* (Wang et al.,
211 2021). The cytoplasm is mainly dominated by smooth endoplasmic reticulum and Golgi
212 apparatus, which is indicative for a non-proteinaceous, and hence likely pheromonal secretion
213 (see further).

214 In spite of the impressive number of glands that can be found in the legs of ants, little is
215 known about their function other than producing lubricants for the leg articulations (Billen,
216 2009). The best documented exceptions are the hindleg glands that produce trail
217 pheromones: the tibial glands in *Crematogaster* (Leuthold, 1968), the pretarsal footprint
218 glands in *Amblyopone australis* (Hölldobler and Palmer, 1989b) and *Stigmatomma reclinatum*
219 (formerly *Amblyopone reclinata*: Billen et al., 2005), and the basitarsal glands in
220 *Onychomyrmex* sp. (Hölldobler and Palmer, 1989a) and *Prionopelta amabilis* (Hölldobler et
221 al., 1992). Another example of a hindleg gland with known function is found in some species
222 of *Diacamma* that reproduce through mated workers, or garmegates. In those species, virgin
223 unmutilated workers attract males with a sex pheromone produced by their metatibial gland in
224 an attempt to mate (Hölldobler et al., 1996; Nakata et al., 1998). The forelegs in many ant
225 species contain a basitarsal ‘antenna cleaner gland’ (Schönitzer et al., 1996), although it
226 remains unclear whether it really plays a role in antennal cleaning. None of the midleg glands,
227 however, has so far been associated with a particular function in any ant species. Even
228 though behavioural observation of *Myopopone castanea* failed to precisely characterize the
229 function of the basitarsal sulcus gland, it narrowed down the possibilities. First, this novel
230 gland could have a cicatrizing function related to LHF, and if so, ants would likely perform LR
231 after larval hemolymph feeding (LHF). However, ants performed LR at a much higher
232 frequency than LHF, and co-occurrence of both was not observed in any of our 10-minute
233 trials, which makes a cicatrizing function unlikely. The two workers seen performing LR and
234 LHF successively also groomed themselves, brood, or a nestmate worker, thus LR could be
235 linked to these particular tasks. Second, LR transferred secretions of the basitarsal sulcus

gland to the gaster, which could suggest a trail pheromone, although trail-laying has never been reported in *M. castanea*. Nonetheless, LR was observed frequently even though workers rarely went outside the nest during our laboratory observations, which weakens such a hypothesis on the function of the basitarsal sulcus gland. In sharp contrast, following LR, workers often groomed and patted the brood, especially the larvae. This observation, along with the ultrastructural characteristics that indicate the elaboration of a non-proteinaceous secretion, suggest that the sulcus gland produces pheromones responsible for nestmate recognition ("colony smell"), possibly cuticular hydrocarbons (CHCs). While an early study suggested that CHCs were produced by the pharyngeal gland in ants (Bagnères and Morgan, 1991), a sequestering rather than secretory function of this gland has become apparent later on (Lucas et al. 2004), and that instead, CHCs are produced by subepithelial glands (Gobin et al., 2003). It is unclear if the basitarsal sulcus gland compensates or supplements the subepithelial gland's secretion. According to the presence/absence of the basitarsal sulcus (Supplementary Table S2) and data provided by Gobin et al. (2003), the two glands co-occur in *Myopopone castanea*, *Myrmecia gulosa*, *M. pyriformis*, and *Nothomyrmecia macrops*, but only the basitarsal sulcus is present in *Myrmecia simillima* and in *M. vindex*. The subepithelial gland is present while the basitarsal sulcus is absent in an undescribed species of *Pseudomyrmex*, and both traits are absent in *Mystrium camillae*, *Prionopelta kraepellini*, and *Stigmatomma reclinatum*. Finally, the occurrence of leg-rubbing behaviour in other ant taxa with and without a basitarsal sulcus gland remains to be investigated.

The distribution of a basitarsal sulcus gland, scattered in two major clades of the ant tree of life, suggests either convergence or single origin followed by multiple losses. Amblyoponinae and Paraponerinae, plus Agroecomyrmecinae, Apomyrminae, Ponerinae, and Proceratiinae form the Poneroid clade, and Myrmeciinae and Pseudomyrmecinae are classified in the Formicoid clade, which also includes the subfamilies Aneuretinae, Dolichoderinae, Dorylinae, Ectatomminae, Formicinae, Heteroponerinae, and Myrmicinae (Borowiec et al., 2020). The relationships among the poneroid subfamilies are still inconclusive (Borowiec et al., 2020), which obscures the degree of relatedness between Amblyoponinae and Paraponerinae. Within Amblyoponinae, the basitarsal sulcus is present in workers and queens of all lineages that make up the XMMAS-clade (*Xymmer*, *Myopopone*, *Adetomyrma*, *Stigmatomma*, and *Fulakora*) save *Mystrium*, and it is absent in the clade *Amblyopone* + *Prionopelta* + *Onychomyrmex* (for the evolutionary history of Amblyoponinae

268 see Ward and Fisher, 2016). Of these, *Onychomyrmex* (Hölldobler and Palmer, 1989a) and
269 *Prionopelta* (Hölldobler et al., 1992; Ito and Billen, 1998) have a gland on the ventrodistal
270 portion of the hindleg basitarsus. This metabasitarsal gland, however, does not display the
271 typical epithelial features nor the external longitudinal sulcus appearance, and therefore
272 cannot be considered homologous to the novel sulcus gland we here describe. An interesting
273 case in this regard is *Stigmatomma luyiae*, which has a round mesobasitarsal sulcus that
274 externally much resembles the metabasitarsal structure of *Onychomyrmex* with similar rod-
275 like cuticular projections (compare figs. 1L and 2G). A study in four amblyoponine genera
276 (*Mystrium*, *Onychomyrmex*, *Prionopelta* and *Stigmatomma*) by Schoeters et al. (1999)
277 revealed that ants of this subfamily, similar to the situation in mutillid wasps, are unique in
278 having a venom gland that lacks a convoluted gland portion. This lacking of a convoluted
279 gland was also confirmed for *Myopopone* (Ito, unpubl. obs.), and together with the variation in
280 the appearance of their sulcus gland illustrates the peculiarity of the subfamily
281 Amblyoponinae. The relationships within the Formicoid clade are well-resolved, and
282 Myrmeciinae and Pseudomyrmecinae have been consistently recovered as sister lineages in
283 phylogenetic inferences (Moreau and Bell, 2013; Branstetter et al., 2017; Borowiec et al.,
284 2020). In fact, Bolton (2003) considered the metabasitarsal sulcus a synapomorphy of the
285 clade formed by these two subfamilies, which he called myrmeciomorph. Noteworthy in this
286 discussion is the predominantly arboreal Dorylinae *Simopone*, whose females in all species
287 present a longitudinal sulcus on the ventral face of the metabasitarsus similar to what is seen
288 in the subfamilies that possess the basitarsal sulcus gland (Bolton and Fisher, 2012); the
289 sulcus is absent in males (Borowiec, 2016). In Myrmeciinae, the basitarsal sulcus is present
290 in the meso- and metabasitarsus in females of *Myrmecia*, *Nothomyrmecia* (Ogata, 1991), and
291 the extinct *Prionomyrmex* (Ward and Brady, 2003). Within Pseudomyrmecinae, a meso- as
292 well as a metabasitarsal sulcus are present in the females of *Myrcidris epicharis* and are
293 completely absent in its sister lineage, *Pseudomyrmex* (Ward, 1990), while most *Tetraponera*
294 species have a metabasitarsal (and often also mesobasitarsal) sulcus in both workers and
295 queens, but not in the males (Ward, 1990).

296 Finally, the novel basitarsal sulcus gland has a remarkable ecological distribution,
297 occurring in arboreal, ground-dwelling as well as subterranean taxa. While Amblyoponinae
298 ants are predominantly cryptic predators in the soil, rotten wood and leaf-litter in tropical and
299 subtropical forests around the world, the neotropical *Paraponera clavata* conspicuously

forages above the ground, from the forest floor to tree canopies, and feeds on a variable diet that includes invertebrates, small vertebrates, carrion, and extrafloral nectar (Brown, 1960; Delsinne et al., 2019, and references therein). The extant Myrmeciinae are distributed in the Australasia region. *Myrmecia* species forage above the ground for nectar and arthropod prey in habitats ranging from deserts to rainforests (Haskins and Haskins, 1950). *Nothomyrmecia macrops* forage on trees for insect prey, also feeding on sugary substances when available in the Mallee Woodlands in southern Australia (Hölldobler and Wilson, 1990). *Myrcidris epicharis* forage on its myrmecophyte hosts *Myrcia madida* and *M. magna* in the Amazon rainforest, and likely feeds on scale insects' exudates (Ferreira and Vasconcelos, 2010; Gaem et al., 2019). *Tetraponera* is widespread in the Old-World tropics, and may forage on vegetation or on ground in habitats that range from urban parks, to deserts, to grasslands, to rainforests, feeding on exudates or preying on other arthropods (Russell et al., 2009; Brian L. Fisher, pers. comm.).

Acknowledgements

We dedicate this paper to the memory of our dear friend and colleague Christian Peeters. We greatly appreciate the assistance of An Vandoren in section preparation for light and electron microscopy and to Alex Vrijdaghs for his help in scanning microscopy. Thanks are also due to Hiroki Miyata for collecting *Onychomyrmex hedleyi* and to Prof. Nguyen Van Sinh (Director Institute of Ecology and Biological Resources, Vietnam), Mr. Nguyen Dac Dai (IEBR), the director and staff of Tay Yen Tu Nature Reserve (Vietnam), and to Dr. Katsuyuki Eguchi (Tokyo Metropolitan University, Japan) for their continuous support. Malagasy specimens would not be accessible without the gracious support of the Madagascar Biodiversity Center team: Balsama Rajemison, Jean-Claude Rakotonirina, Hanitriniana Rasoazanamavo, Nicole Rasoamanana, Njaka Ravelomanana, Manoa Ramamonjisoa, Jean-Jacques Rafanomezantsoa (JJ), Chrislain Ranaivo, Clavier Randrianandrasana, and Bemaheva Fidelisy (Fidel). We also acknowledge the formidable support of Michele Esposito in imaging and databasing ants at the California Academy of Sciences. The research in Vietnam received support through grants for Oversea Research (B 24405010; B16H05769) from JSPS.

References

330 AntWeb, 2020. AntWeb version 8.46.1. California Academy of Science, online at
331 <https://www.antweb.org>. Accessed 14 December 2020.

332 Bagnères, A.G. and Morgan, E.D., 1991. The postpharyngeal glands and the cuticle of
333 Formicidae contain the same characteristic hydrocarbons. *Experientia* 47, 106-111.
334 <https://doi.org/10.1007/BF02041269>

335 Billen, J., 2009. Occurrence and structural organization of the exocrine glands in the legs of
336 ants. *Arthropod Struct. Dev.* 38, 2-15. <https://doi.org/10.1016/j.asd.2008.08.002>

337 Billen, J. and Šobotník, J., 2015. Insect exocrine glands. *Arthropod Struct. Dev.* 44, 399-400.
338 <https://doi.org/10.1016/j.asd.2015.08.010>

339 Billen, J. and Al-Khalifa, M.S., 2016. A novel intramandibular gland in the ant *Brachyponera*
340 *sennaarensis*. *Insect. Soc.* 63, 321-326. <https://doi.org/10.1007/s00040-016-0471-5>

341 Billen, J., Bauweleers, E., Hashim, R. and Ito, F., 2013. Survey of the exocrine system in
342 *Protanilla wallacei* (Hymenoptera, Formicidae). *Arthropod Struct. Dev.* 42, 173-183.
343 <https://doi.org/10.1016/j.asd.2013.01.001>

344 Billen, J., Thys, B., Ito, F. and Gobin, B., 2005. The pretarsal footprint gland of the ant
345 *Amblyopone reclinata* (Hymenoptera, Formicidae) and its role in nestmate recruitment.
346 *Arthropod Struct. Dev.* 34, 111-116. <https://doi.org/10.1016/j.asd.2004.11.003>

347 Billen, J., Lin, C.-C. and Esteves, F.A., 2020. Novel exocrine glands in the foreleg coxae of
348 *Discothyrea* ants. *Arthropod Struct. Dev.* 59, 100981.
349 <https://doi.org/10.1016/j.asd.2020.100981>

350 Bolton, B., 2003. Synopsis and classification of Formicidae. *Mem. Am. Entomol. Inst.* 71, 1-
351 370.

352 Bolton, B. and Fisher, B.L., 2012. Taxonomy of the cerapachyine ant genera *Simopone* Forel,
353 *Vicinopone* gen. n. and *Tanipone* gen. n. (Hymenoptera: Formicidae). *Zootaxa* 3283, 1-
354 101. <https://doi.org/10.11646/zootaxa.3283.1.1>

355 Borowiec, M.L., 2016. Generic revision of the ant subfamily Dorylinae (Hymenoptera,
356 Formicidae). *ZooKeys* 608, 1-280. <https://doi.org/10.3897/zookeys.608.9427>

357 Borowiec, M.L., Rabeling, C., Brady, S.G., Fisher, B.L., Schultz, T.R. and Ward, P.S., 2020.
358 Compositional heterogeneity and outgroup choice influence the internal phylogeny of the
359 ants. *Mol. Phylogenet. Evol.* 134, 111-121. <https://doi.org/10.1016/j.ympev.2019.01.024>

360 Branstetter, M.G., Longino, J.T., Ward, P.S. and Faircloth, B.C., 2017. Enriching the ant tree
361 of life: enhanced UCE bait set for genome-scale phylogenetics of ants and other
362 Hymenoptera. *Methods Ecol. Evol.* 8, 768-776. <https://doi.org/10.1111/20141-210X.12742>

363 Delsinne, T., Sonet, G. and Arias-Penna, T.M., 2019. Subfamilia Paraponerinae. In:
 364 Fernández, F., Guerrero, R.J. and Delsinne, T. (Eds). Hormigas de Colombia.
 365 Universidad Nacional de Colombia, Bogotá, pp. 637-657.
 366 Esteves, F. and Fisher, B., 2016. Taxonomic revision of *Stigmatomma* Roger (Hymenoptera:
 367 Formicidae) in the Malagasy region. Biodivers. Data J. 4, e8032.
 368 <https://doi.org/10.3897/BDJ.4.e8032>.
 369 Ferreira, L.V. and Vasconcelos, H.L., 2010. On a poorly known Amazonian ant-plant
 370 association: *Myrcia madida* McVaugh (Myrtaceae) and *Myrcidris epicharis* Ward
 371 (Hymenoptera: Formicidae: Pseudomyrmecinae). Bol. Mus. Para. Emílio Goeldi Sér.
 372 ciênc. nat. 5, 363-367.
 373 Gaem, P.H., Farroñay, F., Ferreira dos Santos, T., Cabello, N., Mazine, F. and Vicentini, A.,
 374 2019. First record of *Myrcia magna* D.Legrand (Myrtaceae) as a myrmecophyte host for
 375 *Myrcidris epicharis* Ward, 1990 (Formicidae: Pseudomyrmecinae). Sociobiology 66, 592.
 376 <https://doi.org/10.13102/sociobiology.v66i4.4477>.
 377 Gobin, B., Ito, F. and Billen, J., 2003. The subepithelial gland in ants: a novel exocrine gland
 378 closely associated with the cuticle surface. Acta Zool. (Stockholm) 84, 285-291.
 379 <https://doi.org/10.1046/j.1463-6395.2003.00149.x>
 380 Haskins, C.P. and Haskins, E.F., 1950. Notes on the biology and social behavior of the
 381 archaic ponerine ants of the genera *Myrmecia* and *Promyrmecia*. Ann. Entomol. Soc. Am.
 382 43, 461-491. <https://doi.org/10.1093/aesa/43.4.461>
 383 Hölldobler, B. and Palmer, J.M., 1989a. A new tarsal gland in ants and the possible role in
 384 chemical communication. Naturwissenschaften 76, 385-386.
 385 <https://doi.org/10.1007/BF00366215>
 386 Hölldobler, B. and Palmer, J.M., 1989b. Footprint glands in *Amblyopone australis*
 387 (Formicidae, Ponerinae). Psyche 96, 111-121. <https://doi.org/10.1155/1989/70562>
 388 Hölldobler, B., Obermayer, M. and Peeters, C., 1996. Comparative study of the metatibial
 389 gland in ants (Hymenoptera, Formicidae). Zoomorphology 116, 157-167.
 390 <https://doi.org/10.1007/BF02527156>
 391 Hölldobler, B., Obermayer, M. and Wilson, E.O., 1992. Communication in the primitive
 392 cryptobiotic ant *Prionopelta amabilis* (Hymenoptera: Formicidae). J. Comp. Physiol. 170A,
 393 9-16. <https://doi.org/10.1007/BF00195956>
 394 Hölldobler, B. and Taylor, R.W., 1983. A behavioral study of the primitive ant *Nothomyrmecia*
 395 *macrops* Clark. Insect. Soc. 30, 384-401. <https://doi.org/10.1007/BF02223970>

396 Hölldobler, B. and Wilson, E.O., 1990. The Ants. *Cambridge, Mass.: Harvard University*
 397 *Press*, pp. 732.

398 Ito, F., 2010. Notes on the biology of the Oriental amblyoponine ant *Myopopone castanea*:
 399 Queen-worker dimorphism, worker polymorphism and larval hemolymph feeding by
 400 workers (Hymenoptera: Formicidae). *Entomol. Sci.* 13, 199-204.
 401 <https://doi.org/10.1111/j.1479-8298.2010.00384.x>

402 Ito, F. and Billen, J., 1998. Larval hemolymph feeding and oophagy: behavior of queen and
 403 workers in the primitive ponerine ant *Prionopelta kraepelini* (Hymenoptera, Formicidae).
 404 *Belg. J. Zool.* 128, 201-209.

405 Ito, F., Nguyen, D.A., Eguchi, K., Toyota, A. and Billen, J., 2020. Colony composition, queen
 406 behavior, specialized predation on millipedes, and exocrine glands in the ponerine ant
 407 *Myopias conicara* Xu, 1998 (Hymenoptera: Formicidae). *Entomol. Sci.* 23, 142-151.
 408 <https://doi.org/10.1111/ens.12406>

409 Keller, R.A., 2011. A phylogenetic analysis of ant morphology (Hymenoptera: Formicidae)
 410 with special reference to the poneromorph subfamilies. *Bull. Am. Mus. Nat. Hist.* 355,
 411 1-90. <https://doi.org/10.1206/355.1>

412 Leuthold, R.H., 1968. A tibial gland scent-trail and trail-laying behavior in the ant
 413 *Crematogaster ashmeadi* Mayr. *Psyche* 75, 233-248. <https://doi.org/10.1155/1968/73761>

414 Lucas, C., Pho, D.B., Fresneau, D. and Jallon, J.M., 2004. Hydrocarbon circulation and
 415 colonial signature in *Pachycondyla villosa*. *J. Insect Physiol.* 50, 595-607.
 416 <https://doi.org/10.1016/j.jinsphys.2004.04.006>

417 Moreau, C.S. and Bell, C.D., 2013. Testing the museum versus cradle tropical biological
 418 diversity hypothesis: phylogeny, diversification, and ancestral biogeographic range
 419 evolution of the ants. *Evolution* 67, 2240-2257. <https://doi.org/10.1111/evo.12105>

420 Nakata, K., Tsuji, K., Hölldobler, B. and Taki, A., 1998. Sexual calling by workers using the
 421 metatibial glands in the ant, *Diacamma* sp., from Japan (Hymenoptera: Formicidae). *J.*
 422 *Insect Behav.* 11, 869-877. <https://doi.org/10.1023/A:1020816227912>

423 Noirot, C. and Quennedey, A., 1974. Fine structure of insect epidermal glands. *Annu. Rev.*
 424 *Entomol.* 19, 61-80. <https://doi.org/10.1146/annurev.en.19.010174.000425>

425 Ogata, K., 1991. Ants of the genus *Myrmecia* Fabricius: a review of the species groups and
 426 their phylogenetic relationships (Hymenoptera: Formicidae: Myrmeciinae). *Syst. Entomol.*
 427 16, 353-381. <https://doi.org/10.1111/j.1365-3113.1991.tb00694.x>

428 Russell, J.A., Moreau, C.S., Goldman-Huertas, B., Fujiwara, M., Lohman, D.J. and Pierce,
429 N.E., 2009. Bacterial gut symbionts are tightly linked with the evolution of herbivory in
430 ants. *P. Natl Acad. Sci. U.S.A.* 106, 21236-21241.
431 <https://doi.org/10.1073/pnas.0907926106>

432 Schoeters, E., Ito, F., Miyata, H. and Billen, J., 1999. Aberrant venom glands in Amblyoponini
433 (Formicidae, Ponerinae): morphology, ultrastructure and histochemistry. *Acta Zool.*
434 (Stockholm) 80, 3-9. <https://doi.org/10.1046/j.1463-6395.1999.20001.x>

435 Schönitzer, K., Dott, H. and Melzer, R.R., 1996. The antenna cleaner gland in *Messor*
436 *rufitarsis* (Hymenoptera, Formicidae). *Tissue Cell* 28, 107-113.
437 [https://doi.org/10.1016/S0040-8166\(96\)80049-8](https://doi.org/10.1016/S0040-8166(96)80049-8)

438 Wang, C., Steenhuyse-Vandeveld, M., Lin, C.-C. and Billen, J., 2021. Morphology of the
439 novel basimandibular gland in the ant genus *Strumigenys* (Hymenoptera, Formicidae).
440 *Insects* 12, 50.

441 Ward, P.S., 1990. The ant subfamily Pseudomyrmecinae (Hymenoptera: Formicidae): generic
442 revision and relationship to other formicids. *Syst. Entomol.* 15, 449-489.
443 <https://doi.org/10.1111/j.1365-3113.1990.tb00077.x>

444 Ward, P.S. and Brady, S.G., 2003. Phylogeny and biogeography of the ant subfamily
445 Myrmeciinae (Hymenoptera: Formicidae). *Invertebr. Syst.* 17, 361-386.
446 <https://doi.org/10.1071/IS02046>

447 Ward, P.S. and Fisher, B.L., 2016. Tales of dracula ants: the evolutionary history of the ant
448 subfamily Amblyoponinae (Hymenoptera: Formicidae). *Syst. Entomol.* 41, 683-693.
449 <https://doi.org/10.1111/syen.12186>

450

452

453 Fig. 1. Scanning micrographs of anterior face of basitarsus in workers of various *Stigmatomma*
454 species, white arrows indicate the basitarsal sulcus (grey M and H indicate midleg and hindleg
455 basitarsus, respectively). Note absence of sulcus in *S. tsyhady* (D), and circular plate (grey
456 arrow) in *S. luyiae* (L).

457

458
459
460
461
462

Fig. 2. **A.** Profile view of *Myropopone castanea* worker with arrows indicating sulcus on both midleg and hindleg basitarsus. **B-G.** Scanning micrographs of anterior face of basitarsus in various amblyoponine workers, white arrows indicate sulcus (grey M and H indicate midleg and hindleg basitarsus, respectively). Note oval basitarsal sulcus indicated by grey arrow in *Onychomyrmex doddi* (G), with close-up view in inset.

463

464 Fig. 3. Scanning micrographs of anterior face of basitarsus in workers of non-Amblyoponini
 465 species, white arrows indicate basitarsal sulcus (grey M and H indicate midleg and hindleg
 466 basitarsus, respectively).

467

468 Fig. 4. Semithin sections through the worker basitarsus (frontal in image D, longitudinal in G,
 469 transverse in all other images), showing the sulcus (S) and the epithelial basitarsal sulcus gland
 470 (SG). **A.** *Stigmatomma roahady* midleg. **B.** *S. roahady* hindleg. **C.** *Paraponera clavata* midleg.
 471 **D.** *Myopopone castanea* midleg. **E.** *Tetraponera rufonigra* hindleg. **F.** *Nothomyrmecia macrops*
 472 hindleg. **G.** *Onychomyrmex hedleyi* hindleg. obs: oval basitarsal sulcus, BG: basitarsal gland.

473

474 Fig. 5. Electron micrographs of the basitarsal sulcus gland. **A.** Overview of gland epithelium
 475 (SG) underneath cuticle (ct) of *Stigmatomma roahady* midleg. Inset shows detail of cuticle
 476 surface inside sulcus with minuscule cuticular depressions (arrowheads). **B.** Apical cytoplasm
 477 of *Paraponera clavata* hindleg gland with microvillar border (mv). **C.** Detail of cytoplasm in
 478 *Stigmatomma roahady* midleg gland, showing smooth (SER) and granular endoplasmic
 479 reticulum (RER), well-developed Golgi apparatus (GA) and mitochondria (M). **D.** Central
 480 cytoplasm of *Myopopone castanea* hindleg gland showing extensive smooth endoplasmic
 481 reticulum (SER) and mitochondria (M). **E.** Basal cytoplasm of *M. castanea* hindleg gland with
 482 prominent basal invaginations (bi). bm: basement membrane, N: nucleus.

Fig. 6. Leg-rubbing behaviour (LR) of *Myopopone castanea*. **A.** Lateral leg-rubbing (LLR) involves four legs and the gaster tip while the two remaining legs support the body. **B.** Posterior leg-rubbing (PLR) only involves the hindlegs, which are rubbed below the gaster while the ant is standing. Circles highlight the LR.

Supplementary file legends

Supplementary Video 1: Leg-rubbing (LR) behaviour in *Myopopone castanea*. The worker in the top right corner performs posterior (PLR) and lateral (LLR) leg-rubbing.

Supplementary Video 2: Slow-motion playback of lateral leg-rubbing (LLR) in *Myopopone castanea*. Basitarsi and tarsomeres touch during this behaviour.

Supplementary Table S1: Specimen and collection data of material examined in this study.

Supplementary Table S2: Distribution of the basitarsal sulcus in the specimens examined in this study.

Supplementary Table S3: *Myopopone castanea* behavioural observation matrix.