Sociobiology 61(4): 386-392 (December 2014) DOI: 10.13102/sociobiology.v61i4.386-392

Sociobiology
An international journal on social insects

RESEARCH ARTICLE - BEES

Exocrine glands in the legs of the stingless bee *Frieseomelitta varia* (Lepeletier) (Apidae: Meliponini)

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Article History

Edited by
Denise Araujo Alves, ESALQ-USP, Brazil
Received 30 September 2014
Initial acceptance 25 October 2014
Final acceptance 30 October 2014

Keywords
Legs, exocrine glands, morphology, Meliponini.

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Abstract

Social insects are known for their overwhelming diversity of exocrine glands. This study examines the glands in the legs of workers of *Frieseomelitta varia* (Lepeletier). A variety of 15 glands was found, with glands occurring in every leg segment, whereas previous studies only described 5 glands in stingless bee legs. Six glands are novel exocrine structures for social insects. Glands occurring in the articulation region between adjacent leg segments may occur in a repetitive pattern, and probably have a lubricant function. For most glands, however, the function is not yet known, and will require further experimental work.

Introduction

Social insects display an enormous variety of exocrine glands, the secretions of which play multiple roles in their social organization (Hölldobler & Wilson, 1990; Billen & Morgan, 1998; Billen, 2011). Following their cellular organization, all glands can be classified in one of two major groups as first described in the pioneer paper on insect exocrine glands by Noirot and Quennedey (1974): class-1 glands are formed by simple epithelial secretory cells, while class-3 glands are formed by a number of bicellular units, each unit comprising a large spherical secretory cell and an accompanying slender duct cell. The ducts and the corresponding pores at their opening site have a characteristic diameter of 0.5-1 µm. Glands of both groups can either occur underneath the external cuticle, and in this case release their secretion directly to the exterior, or they can be part of an invaginated reservoir sac, where secretion can be temporarily stored. This leads to five anatomical types (Fig 1).

The various glands are not only confined to the head, thorax and abdomen, but can also be found in the appendages such as the antennae, mouthparts and legs. In ants, an astonishing variety of 20 leg glands has been described (Billen, 2009). Bees, as well, have several glands in their legs, and especially stingless bees have been studied the most (Cruz-Landim et al., 1998). The latter study presented data on leg glands in the three castes of 13 meliponine species belonging to 7 genera. Its most important conclusions were the occurrence of epithelial glands only in the basitarsus and pretarsus (with a report that queens have sacculiform glands also in their femur), the widespread occurrence of class-3 glandular cells in the various leg segments, and that tarsomeres 2 to 4 did not contain any gland cells (Cruz-Landim et al., 1998, updated in Cruz-Landim, 2002). Our present study deals with workers of *Frieseomelitta varia* (Lepeletier), a species that was not included in the survey study by Cruz-Landim et al. (1998), but which revealed the occurrence of several other leg glands, that therefore can be considered as novel exocrine structures.

Material and methods

Both callow and foraging workers of *F. varia* were collected from natural colonies at the apiary of the Universidade de São Paulo campus in Ribeirão Preto, SP, Brazil. For the histological work, we used forager workers only (n=3), as they are relatively old. This is important to recognize epithelial glands, as callow workers may display a thick tegumental epithelium because of cuticle formation at their young age. As

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ISSN: 0361-6525

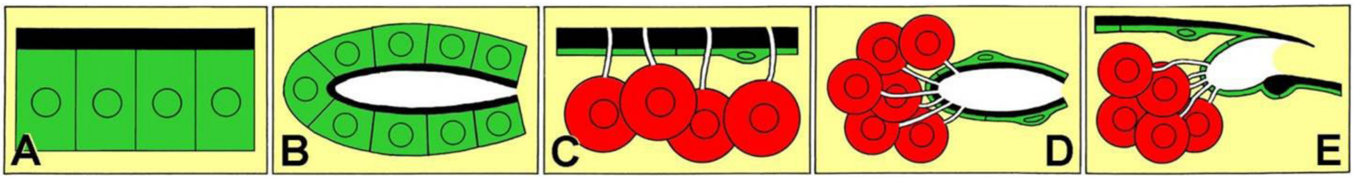

Fig 1. General gland types, with types A and B formed by epithelial class-1 glandular cells, types C, D and E formed by bicellular secretory units containing class-3 gland cells. Gland types A and C have no reservoir and release their secretion directly to the exterior, types B, D and E have a reservoir for temporary storage of secretion. This reservoir can be formed by an invagination of an intersegmental membrane, see type E (from Billen, 2009a).

cuticle formation is completed in foragers, a thick epithelium in such older individuals can be considered as glandular tissue. In order to allow proper penetration of fixative and the various chemicals used for dehydration and embedding, fore-, mid- and hindlegs were cut into smaller pieces by making cross cuts in the femur, tibia and basitarsus. Tissues were fixed in cold 2% glutaraldehyde, buffered with 50 mM Na-cacodylate and 150 mM saccharose, followed by postfixation in 2% osmium tetroxide. After dehydration through a graded acetone series, tissues were embedded in Araldite resin. Serial semithin sections with a thickness of 1 µm were made with a Leica EM UC6 ultramicrotome, stained with methylene blue and thionin, and examined with an Olympus BX-51 light microscope. For scanning microscopy, however, we only used callow workers (n=3), as they have clean legs whereas forager legs may be dirty and/or covered with resin material. Legs were mounted on stubs, gold coated, and viewed in a JEOL JSM-6360 scanning microscope.

Results and discussion

The various exocrine leg glands will be presented from most proximal (coxa) to most distal (pretarsus). Figure 2 and Table 1 give a survey of the occurrence of the various glands. All photographs are shown with the dorsal side up; all sections are longitudinal, the proximal side is always to the left.

1. *Coxal gland*

The distal region of the coxae of the three leg pairs contains a few rounded class-3 cells with a diameter around 30 µm. Their ducts open through the articulation membrane with the trochanter (Fig 3A). Similar coxal glands have also been described in ants (Schoeters & Billen, 1993), and because of their opening through the articulation membrane, they possibly have a lubricant function. Cruz-Landim et al. (1998) and Cruz-Landim (2002) also report the existence of class-3 glandular cells in workers of several meliponine species, but in the proximal dorsal part of the coxae, and with unknown opening site of the ducts.

2. *Coxal epithelial gland*

Besides the class-3 coxal gland cells, the coxae of the three leg pairs also contain a glandular epithelium with a thickness between 10 and 15 µm (Fig 3A, B), that occurs as a kind of distal belt along the entire circumference of the coxae. The glandular epithelium is distinctly different from the much thinner proximal coxal epithelium. This gland was not mentioned in the review papers on meliponine leg glands by Cruz-Landim et al. (1998) and Cruz-Landim (2002), and it is also different from the epithelial basicoxal gland in ants, that occurs dorsally in the proximal region of the mid- and hindlegs of poneromorph ants (Billen & Ito, 2006). It therefore represents a novel exocrine structure for social insects with currently unknown function.

3. *Distal trochanter gland*

The distal part of the trochanter of the three leg pairs contains a ventral glandular epithelium with a thickness around 10 µm (Fig 3C). An epithelial trochanter gland has also been described in several ant species, but occurs in the proximal ventral part only and it is presumed to have a lubricant function (Billen, 2008). Cruz-Landim et al. (1998) and Cruz-Landim (2002) do not mention about an epithelial gland in the trochanter of Meliponini, but instead report the occurrence of class-3 gland cells in the dorsodistal region near the articulation with the coxa, where their ducts open through the external cuticle (Cruz-Landim, 2002). Our study of *Frieseomelitta varia*, however, did not show any class-3 gland cells in the trochanter.

4. *Proximal femoral gland*

Sections through the proximal part of the hindleg femur revealed the occurrence of a linearly arranged cluster of class-3 cells that are wedged in between the extensive femoral musculature (Fig 3D). The cells appear rather square on sections and measure approx. 50x20 µm. We did not find similar cells in the fore- and midlegs. The ducts open through the external dorsal cuticle (arrow in Fig 3D), where they can also be seen with scanning microscopy (Fig 3E). A similar proximal femoral gland was also reported, albeit without information in which leg pairs, in all meliponine species investigated by Cruz-Landim et al. (1998). The function of this gland remains unknown.

5. *Dorsodistal femoral gland*

The dorsodistal region of the femur in the three leg pairs contains a cluster of approx. 10 rounded class-3 glandular cells that have a diameter around 25 µm (Fig 3F). Their

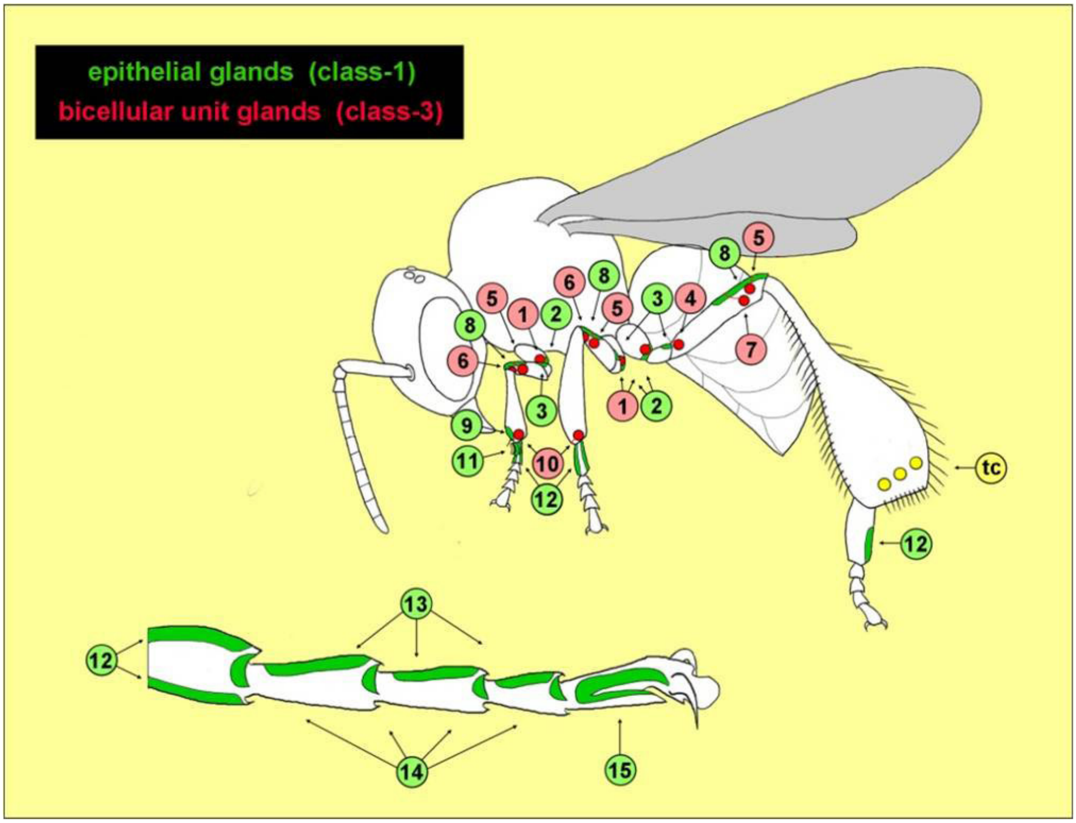

Fig 2. Schematical survey of the various exocrine glands in the legs of *Frieseomelitta varia* workers. Gland numbers correspond with Table 1 and the main text. The anatomical organization in the distal tarsomeres is shown in the detail drawing. tc = undefined tibial cells in the hindlegs.

ducts open through the external cuticle (arrows in Fig 3F), and can also be recognized with scanning microscopy (Fig 3G). As their opening site is not directly associated with the articulation membrane with the tibia, a lubricant function is less probable. Cruz-Landim et al. (1998) and Cruz-Landim (2002) do not mention about this gland in Meliponini, while also ants do not have it (Billen, 2009). Scattered class-3 glandular cells and pores of the corresponding duct cells at the femur-tibia junction, and especially in the various tarsomeres, were found in the wasp *Polistes dominulus* (Christ, 1791), and are probably involved in pheromone production (Beani & Calloni, 1991).

6. *Distal femoral gland*

The distal region of the femur also contains, at least in the fore- and midleg, a few ovoid class-3 glandular cells measuring approx. 30 by 20 µm (Fig 4A). Their ducts open through the articulation membrane with the tibia, which is probably an indication of a lubricant function. Similar gland cells have been reported in ants (Billen, 2009), but were not yet listed for Meliponini (Cruz-Landim et al., 1998; Cruz-Landim, 2002).

7. *Ventrodistal femoral gland*

Besides the dorsodistal femoral gland (which occurs dorsally and opens externally at the tip of the femur, see under 5) and the distal femoral gland (which occurs centrally and opens through the intersegmental membrane, see under 6), the distal femur of the hindleg contains a ventrally located cluster of numerous class-3 glandular cells. Contrary to the dorsodistal femoral gland, however, this 'ventrodistal femoral gland' has long ducts that run posteriorly in an upward direction to open eventually at the dorsolateral femoral surface (Fig 4C). The scattered ducts are clearly visible with scanning microscopy (Fig 4B). As this gland was not observed in other stingless bees by Cruz-Landim et al. (1998) and Cruz-Landim (2002), and it is also lacking in ants (Billen, 2009), it can be considered a novel exocrine gland for social insects. As the opening site of the ducts is quite far from the junction with the tibia, a lubricant function is not very probable.

8. *Dorsodistal epithelial femoral gland*

In all three leg pairs, the femur also contains underneath its dorsodistal region a glandular epithelium with a thickness around 20 µm (Figs 4A, 4C). The occurrence of this gland was not noticed in other Meliponini (Cruz-Landim et al., 1998; Cruz-Landim, 2002), nor does it occur in ants (Billen, 2009). The function of this novel gland remains unknown. Cruz-Landim et al. (1998) found an internal sacculiform epithelial gland inside the femur of a few stingless bee species, but only in the queens, and without information of where this sac-like gland opens to the outside.

9. *Dorsodistal epithelial tibial gland*

The dorsodistal region of the tibia in the forelegs contains a glandular epithelium with a thickness around 10 µm (Fig 4D). This gland was not found in other Meliponini (Cruz-

Table 1. Survey of the various leg glands in workers of *Frieseomelitta varia*, and their occurrence in the three leg pairs. Gland names shown in bold represent exocrine structures that have not been reported before for social insects. Gland numbering is the same as in Figure 2 and in the main text, gland type corresponds with Figure 1. + indicates gland presence, - indicates absence. The right column 'CL' indicates whether or not the occurrence of a gland was reported in the 1998 review article on leg glands in Meliponini bees by Cruz-Landim et al. and the same author's book chapter update in 2002.

Nr	Gland	type	foreleg	midleg	hindleg	first reference	CL
1	Coxal gland	E	+	+	+	Schoeters & Billen, 1993	yes
2	**Coxal epithelial gland**	A	+	+	+	This paper	no
3	**Distal trochanter gland**	A	+	+	+	This paper	no
4	Proximal femoral gland	C	-	-	+	Cruz-Landim et al., 1998	yes
5	Dorsodistal femoral gland	C	+	+	+	Beani & Calloni, 1991	no
6	Distal femoral gland	E	+	+	-	Billen, 2009	no
7	**Ventrodistal femoral gland**	C	-	-	+	This paper	no
8	**Dorsodistal epithelial femoral gland**	A	+	+	+	This paper	no
9	**Dorsodistal epithelial tibial gland**	A	+	-	-	This paper	no
10	Dorsodistal tibial gland	C	+	+	-	Cruz-Landim et al., 1998	yes
11	Antenna cleaner gland	A	+	-	-	Schönitzer et al., 1996	no
12	Epithelial basitarsal gland	A	+	+	+	Cruz Landim & de Moraes, 1994	yes
13	**Epithelial tarsomere glands**	A	+	+	+	This paper	no
14	Distal tarsomere glands	A	+	+	+	Billen et al., 2000	no
15	Arolium gland	B	+	+	+	Arnhart, 1923	yes

Landim et al., 1998; Cruz-Landim, 2002), nor in ants (Billen, 2009), and thus forms another novel exocrine structure for social insects. Its function remains as yet unknown.

10. *Dorsodistal tibial gland*

In the dorsodistal region of the fore- and midlegs, we found some large rounded class-3 glandular cells with a diameter around 40 µm (Fig 5B). Their ducts open through the upper external cuticle (Fig 4E), which make this gland a tibial equivalent of the dorsodistal femoral gland (see under 5). Similar glandular cell clusters have also been described in other stingless bee species (Cruz-Landim et al., 1998; Cruz-Landim, 2002), as well as in ants (Billen, 1997). As for the dorsodistal femoral gland, a lubricant function seems not to be very likely as the duct openings are situated on the external surface instead of opening through the articulation membrane.

Undefined tibial cells

As in the majority of most other bees, the hindleg tibia of *Frieseomelitta varia* workers is considerably enlarged as it forms part of the pollen collecting apparatus (Fig 2). Inside the distended part of the tibia, we found a massive number of large ovoid to polygonal cells of 40-50 µm with a rounded nucleus and highly vacuolar cytoplasm (Fig 4F). Their initial appearance reminds of duct cells, but we could not find any ducts, neither on sections nor under the scanning microscope. Following the description and classification of the insect fat body as given in the review paper by Roma et al. (2010), they may be oenocytes or trophocytes, although it remains unclear why they are so numerous in the hindleg tibia.

11. *Antenna cleaner gland*

A common characteristic for most Hymenoptera is the occurrence of an antenna cleaning apparatus on both forelegs, that consists of a prominent tibial spur and a semicircular comb-like differentiation of the proximal basitarsus (Fig 5A). The tegumental epithelium underneath the basitarsal comb is formed by cylindrical cells with a height around 40 µm (Fig 5B). A major part of this complex structure is formed by nervous tissue, as sensillar structures extend into the comb cuticle (Fig 5B). The other part probably forms part of the antenna cleaner gland that was first described in the ant *Messor rufitarsus* (Fabricius) by Schönitzer et al. (1996). In spite of its name, however, no experimental evidence exists for a direct function of this gland in the process of antennal cleaning. No mention of this gland in Meliponini was made in the review papers by Cruz-Landim et al. (1998) and Cruz-Landim (2002).

12. *Epithelial basitarsal gland*

The epithelium of the entire (fore- and midleg: Fig 5C) or dorsal part of the basitarsus (hindleg: Fig 5D) is differentiated into a thickened glandular tissue. The existence of this epithelial basitarsal gland was first reported for a few meliponine species and for the honeybee (Cruz Landim & de Moraes, 1994). Its function as yet remains unknown.

13. *Epithelial tarsomere glands*

The entire dorsal epidermis of the three intermediary tarsomeres t2-t3-t4 in the three leg pairs appear as a clear glandular epithelium with a thickness of up to 15 µm, whereas the ventral side shows a squamous epithelium of hardly 1-2 µm (Fig 5E). These conspicuous epithelial glands have never been described

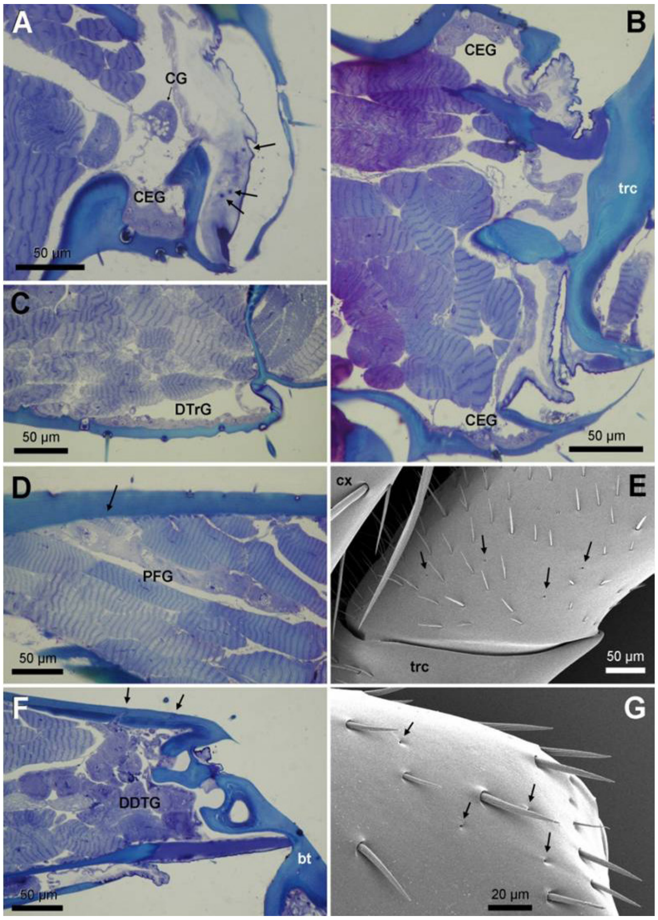

Fig 3. **A**. Distal region of midleg coxa with coxal epithelial gland (CEG) and coxal gland (CG). Arrows indicate ducts of the coxal gland penetrating the articulation membrane with the trochanter. **B**. Mediodistal region of midleg coxa and articulation with trochanter (trc), showing belt-like coxal epithelial gland (CEG). **C**. Distal region of midleg trochanter with distal trochanter gland (DTrG). **D**. Proximal region of hindleg femur with proximal femoral gland (PFG), arrow shows duct penetrating cuticle. **E**. Scanning micrograph of proximal hindleg femur, arrows indicate opening of proximal femoral gland ducts. **F**. Distal region of midleg tibia and articulation with basitarsus (bt). Arrows indicate opening of ducts of the dorsodistal tibial gland (DDTG) through the cuticle. **G**. Scanning micrograph of distal part of foreleg tibia, arrows indicate duct openings of dorsodistal tibial gland. cx: coxa.

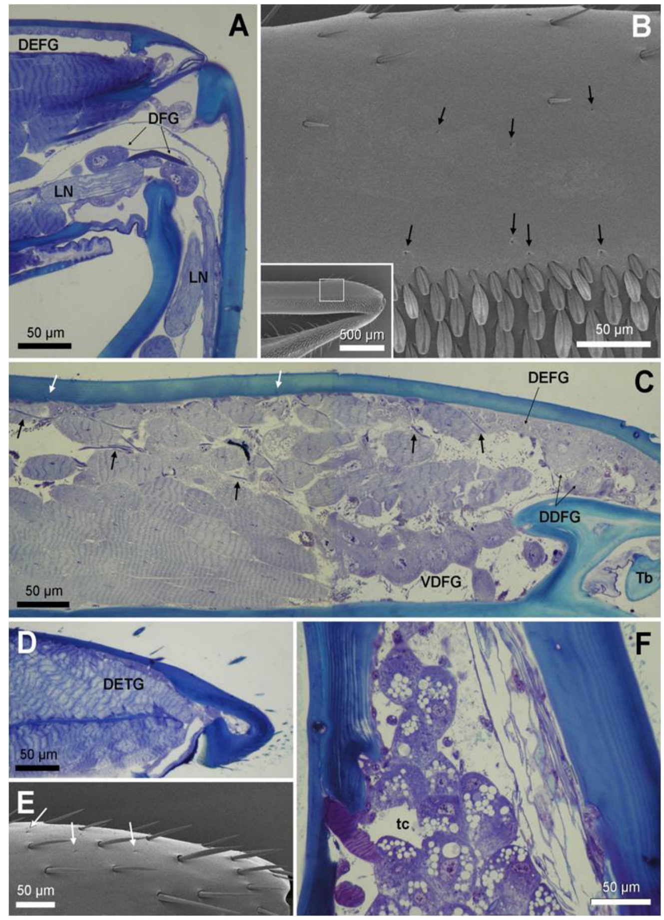

Fig 4. **A**. Junction between midleg femur and tibia, showing the dorsodistal epithelial femoral gland (DEFG) and distal femoral gland (DFG). **B**. Scanning micrograph of hindleg femur, arrows indicate duct openings of the dorsodistal femoral gland (inset shows region where picture is taken). **C**. Section through distal region of hindleg femur. Arrows indicate long ducts of the ventrodistal femoral gland (VDFG; white arrows show ducts penetrating cuticle). **D**. Distal part of foreleg tibia with dorsodistal epithelial tibial gland (DETG). **E**. Scanning micrograph of foreleg tip, arrows indicate duct openings of the dorsodistal tibial gland. **F**. Distal region of hindleg tibia with numerous probably non-glandular tibial cells (tc). DDFG: dorsodistal femoral gland, LN: leg nerve, Tb: tibia.

previously, also not in other Meliponini (Cruz-Landim et al., 1998; Cruz-Landim, 2002). In ants, a glandular differentiation of the dorsal tarsomere epithelium only occurs in the proximal region, where it forms part of the articulation with the previous tarsomere, probably indicating a lubricant function (Billen, 1997, 2009). The glandular nature of the entire tarsomere upper surface, as now described for *F. varia*, however, can hardly be linked with any lubricating role.

14. *Distal tarsomere glands*

In the three leg pairs, tarsomeres t1-t2-t3-t4 show a semicircular mediodistal glandular epithelium with a thickness of approx. 20 µm (Fig 5E). Their repetitive occurrence and their location in the articulation region with the adjacent tarsomere support a lubricant function. These distal tarsomere glands have not been reported for other Meliponini species (Cruz-Landim et al., 1998; Cruz-Landim, 2002), but do exist in ants (Billen et al., 2000).

15. *Arolium gland*

The pretarsus (= tarsomere t5) in the three leg pairs contains the conspicuous arolium gland, which is formed by a glandular epithelium with a thickness of up to 30 µm (Fig 5E). The lumen of the gland is continuous with the interior arolium. The reservoir sac is ventrally penetrated by the leg tendon, which in turn makes contact with the ventral wall of the arolium. Although there is a transverse ventral groove in this region, there is no direct contact between the lumen of the arolium gland and the exterior (Fig 5E). The arolium gland was first described almost a century ago by Arnhart (1923) in the honeybee, and it is the most common leg gland, that is present in all social Hymenoptera (bees: Arnhart, 1923; Cruz Landim & Staurengo, 1965; bumblebees: Pouvreau, 1991; wasps: Billen, 1986; ants: Billen, 2009). Initially, the gland secretion was thought to play a role in the adhesion to smooth surfaces (Arnhart, 1923; Pouvreau, 1991), which is very doubtful as there is no contact between the gland lumen and the exterior. Instead, gland secretion can be pumped into the arolium and thus probably acts as a hydraulic system, with the resulting changes in arolium shape responsible for adhesion (Federle et al., 2001). The eventual role in substrate marking (Goulson et al., 2000) may not be attributed to the arolium gland for the same anatomical reason, but has been shown to be the result of secretions from femoral and tibial tendon glands, that are deposited at the pretarsus ventral surface through the leg tendon (Jarau et al., 2004, 2005). These femoral and tibial tendon glands, elegantly described by Jarau et al. (2004) in the three leg pairs of *Melipona seminigra* Friese, were not found in our study of *F. varia*.

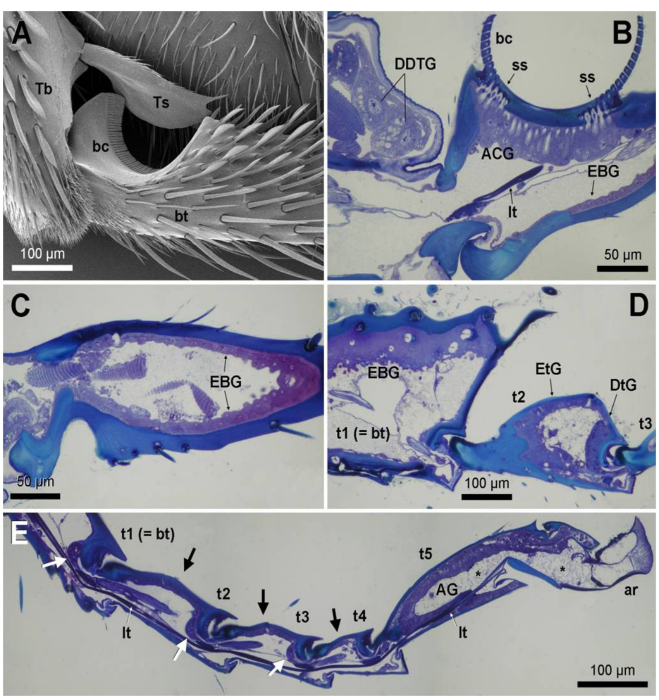

Fig 5. **A**. Scanning micrograph of the antenna cleaning apparatus at the junction between the foreleg tibia (Tb) and basitarsus (bt), which is formed by the tibial spur (Ts) and a basitarsal comb (bc). **B**. Section through the foreleg tibia-basitarsus junction with indication of the dorsodistal tibial gland (DDTG), antenna cleaner gland (ACG) and epithelial basitarsal gland (EBG). **C**. Foreleg basitarsus with epithelial basitarsal gland (EBG). **D**. Hindleg basitarsus and second tarsomere (t2), showing the epithelial basitarsal gland (EBG), epithelial tarsomere gland (EtG) and distal tarsomere gland (DtG). **E**. Section through foreleg tarsomeres with the arolium gland (AG), epithelial tarsomere glands (black arrows) and distal tarsomere glands (white arrows). ar: arolium, lt: leg tendon, ss: sensillar structures, t1-t5: tarsomeres 1-5. The arolium gland lumen is marked with asterisks (*).

Conclusion

The legs of *F. varia* workers represent an impressive glandular environment with 15 different glands, of which 6 are novel exocrine structures for social insects in general. Earlier work documenting the leg glands of 13 stingless bee species (Cruz-Landim et al., 1998; Cruz-Landim, 2002) listed only one third of the glands we have found in *Frieseomelitta*. This may be due to a less systematic approach previously, as we studied semithin serial sections of 1 µm thickness, whereas Cruz-Landim et al. (1998) worked with 6 µm sections. We found glands in all leg segments, which often show a similar pattern in the three leg pairs. Although difficult to prove experimentally, several glands probably have a function in the production of lubricant substances, especially when their opening site is situated at the junction between adjacent segments, while they may also occur in a repetitive pattern such as the distal tarsomere glands. Also the arolium gland as part of a hydraulic system that regulates arolium shape and hence adhesion to the substrate (Federle et al., 2001) and the antenna cleaner gland have mechanical functions, although empirical evidence for the latter is not yet clear. Other leg glands may be involved in pheromone production. Ants are substrate-bound and therefore often rely on trail pheromones, for which several hindleg glands have been shown as the anatomical source (Billen, 2009). Wasps have been reported to use leg glands for scent marking as well (Beani & Calloni, 1991), while Lensky & Slabezki (1981) already reported how honeybee queens inhibit the construction of royal cells with 'foot-print pheromones'. The only known example in meliponine bees of leg glands having a pheromonal function is the food marking in *M. seminigra* with secretions from the femoral and tibial tendon glands (Jarau et al., 2004). Further behavioral and chemical work in future will hopefully reveal more insight in the role of the multiple glands that are present in the legs of stingless bees.

Acknowledgements

We are very grateful to An Vandoren for her help with section preparation, to Alex Vrijdaghs for his assistance with scanning microscopy, and to Denise Araujo Alves for providing us with the bees.

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