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Survey of the exocrine system in *Protanilla wallacei* (Hymenoptera, Formicidae)

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ABSTRACT

We studied the exocrine system of both workers and ergatoid queens of *Protanilla wallacei* using light, scanning and transmission electron microscopy. Our survey revealed the presence of 26 glands, of which 6 had never been found before in ants. Five of these represent novel discoveries for social insects in general. The overall novel discoveries comprise an epithelial stipes gland, a pharyngeal wall gland, a central petiole gland, a lateral postpetiole gland and a foot-sole gland in the hindleg pretarsi. The intramandibular epithelial gland was already reported in some bees previously, but is now for the first time also reported in ants. The exocrine system of workers and ergatoid queens is very similar, with only the spermathecal gland showing an obvious difference. This is in line with the limited anatomical as well as behavioural difference between both castes in *Protanilla* compared to the situation in *Leptanilla*.

1. Introduction

It is generally known that ants represent one of the most ubiquitous terrestrial arthropods, yet species of some groups are only exceptionally encountered. This is especially true for members of the subfamily Leptanillinae, of which only a few tens of species have been described so far (Bolton, 1995). Among the reasons for their elusive nature are the very small specimen size (workers often measure less than 2 mm), the small colony size (usually less than 200 workers: Masuko, 1990), and their often hypogaeic and army ant-like lifestyle (Masuko, 1990). They do not have a close doryline affinity, however (Bolton, 1990), and phylogenetically form a very basal lineage that is the sister group to all other ants (Moreau et al., 2006; Ward, 2007; Kück et al., 2011). The difficulty to find live colonies of these ants is also reflected in the scarcity of information on their general biology and social organization. The few papers on Leptanillinae that are available deal with the genus *Leptanilla*, and report on the peculiar larval hemolymph feeding behaviour of the queen and the corresponding unique larval morphology (Wheeler and Wheeler, 1988; Masuko, 1989), a description of the thoracic and abdominal glands (Hölldobler et al., 1989), and of the mandibular gland by Billen et al. (1998).

The fortunate availability of live workers as well as ergatoid queens of *Protanilla wallacei* allowed us to study the various exocrine glands of this species, which to our knowledge is the first such report for this ant genus and for the tribe Anomalomyrmini to which they belong. It moreover reports on the discovery of six glands, that had never been found before in ants, five of which are novel for social insects altogether.

2. Material and methods

The *Protanilla* here studied belongs to a formally undescribed species, that was already mentioned in "The Ants" by Hölldobler and Wilson (1990: page 592; Fig. 16–18) as *P. wallacei*. The species is currently under description by Robert Taylor (pers. comm.). During field work in Ulu Gombak, peninsular Malaysia, by one of us (FI), a colony of *P. wallacei* (colony code FI11-96) was found in a dead broken twig on the forest floor. The sample contained 17 ergatoid queens, 25 workers and 29 larvae. They could be kept alive for a few weeks by feeding them *Occasjapyx* diplurans, which allowed some simple behavioural observations such as prey conquest and trail following. Details of their ecological and behavioural characteristics will be reported in a forthcoming publication. Voucher specimens are deposited at FRIM (Forest Research Institute Malaysia, Kepong, Malaysia).

The head, prothorax, posterior thorax, petiolar region and abdomen of 3 queens and 4 workers were fixed in cold 2% glutaraldehyde, buffered at pH 7.3 with 50 mM Na-cacodylate and

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150 mM saccharose. Postfixation was carried out in 2% osmium tetroxide in the same buffer. After dehydration in a graded acetone series, tissues were embedded in Araldite and sectioned with a Leica EM UC6 ultramicrotome. Serial semithin sections with a thickness of 1 μm were stained with methylene blue and thionin and viewed in an Olympus BX-51 microscope. Double stained 70 nm thin sections were examined in a Zeiss EM900 electron microscope. Specimens for scanning microscopy were critical point dried in a Balzers CPD 030 instrument and examined in a JEOL JSM-6360 scanning microscope.

3. Results and discussion

The following paragraphs report on the various glands we found in *P. wallacei* (Fig. 1), from the anterior tip of the head towards the posterior end of the abdomen. All figures of longitudinal sections are shown with the anterior side to the left. For some glands, also ultrastructural data are provided, for others this was not possible. This was due to the difficulty with these tiny ants to interrupt the serial sectioning for light microscopy at the very site of a particular gland in order to have material available for electron microscopy. The following descriptions nevertheless provide the most comprehensive information available so far on the exocrine system in the elusive representatives of the subfamily Leptanillinae, combining light microscopy, transmission and scanning electron microscopy.

A first gland, that is not confined to a particular body part, is the **subepidermal gland**, that occurs over the entire body surface (Gobin et al., 2003). According to the standard classification of Noirot and Quennedey (1974), the gland is formed by single units of class-3 secretory cells and their accompanying duct cells, the latter opening at the body surface as a tiny isolate pore with a diameter of approx. 0.5–1 μm (Fig. 2A–C). We found it in both workers and queens, with pores occurring on the head, thorax, petiole and postpetiole, as well as the abdomen. No function could yet be attributed to the widespread subepidermal gland, though it could contribute to the composition of the epicuticular coating (Gobin et al., 2003). A conspicuous zone with an abundance of concentrated pores was found on the anterior part of the first gastral tergite (Fig. 2C), although we unfortunately did not have this body region available for proper histological checking. We therefore cannot yet conclude whether this clustered occurrence of pores on the first gastral segment corresponds with an eventual novel gland or not.

3.1. Cephalic glands

The head harbours a variety of glands that are associated with the mouthparts and the pharynx (survey of a longitudinal head section in Fig. 2D). Inside the mandibles of both workers and queens, we found an **intramandibular epithelial gland**, that has not been reported in ants previously, though it has been reported for some stingless bees (Costa Leonardo, 1978). The epithelium has a thickness of approx. 30 μm and lines the proximal part of the ventral mandibular cuticle (Fig. 2E). Its ventral position close to the mouth may be indicative for a function related to food processing. Intramandibular gland cells of class-3, however, which are fairly common among ants (Schoeters and Billen, 1994), were not found.

The paired **mandibular gland** at each side consists of a cluster of secretory cells of class-3, that open through their accompanying duct cells in the reservoir, that occurs laterally in the anterior portion of the head. Workers have 9 rounded secretory cells at each side, with an average diameter of 37.4 ± 4.4 μm, queens have 12 cells per side, measuring 28.0 ± 3.1 μm (Fig. 2F). Each reservoir narrows when approaching the mandibular base, into which it opens through a conspicuous T-shaped structure (Fig. 2G). This is in agreement with the situation in other ants (Grasso et al., 2004), which enables mechanical release of the glandular products when the mandibles are opened. In many ants, the mandibular gland is involved in alarm-defence behaviour, which is probably also the

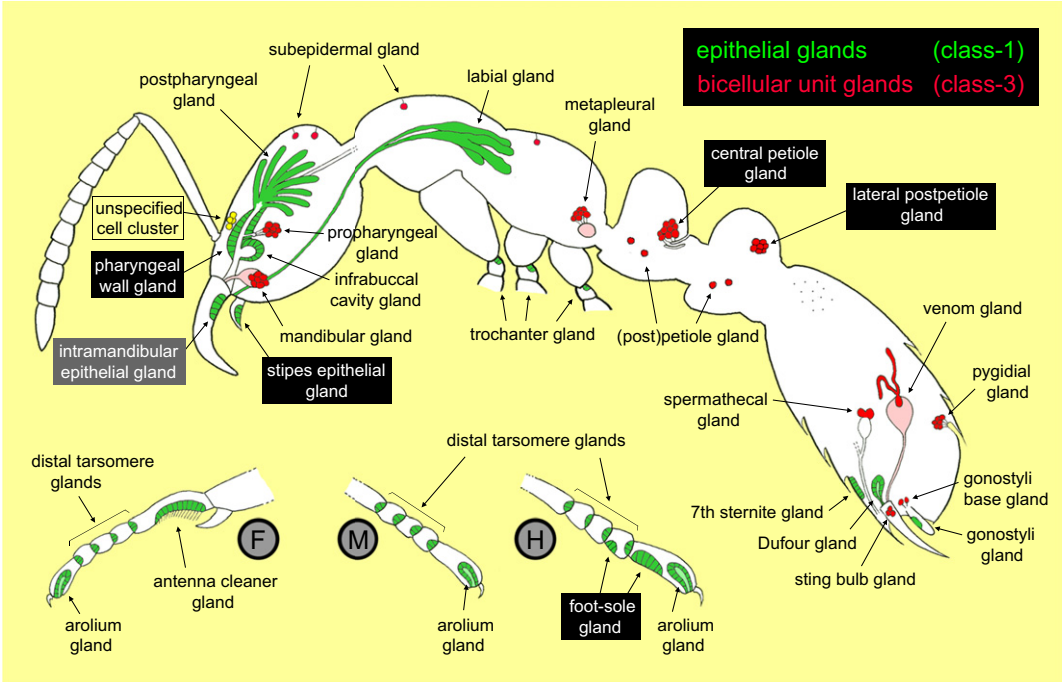


Fig. 1. Profile of *Protanilla wallacei* and the distal part of the legs with indication of the various exocrine glands. The gland names shown in white lettering on dark background are new reports among the Formicidae, of which the 5 glands on black background are novel for social insects in general. The stippled area in the first gastral segment corresponds with the occurrence of numerous pore openings, that possibly represent class-3 glands. As no material for proper histological confirmation was available, we do not yet want to assign a gland name to this structure. F: foreleg, M: midleg, H: hindleg.

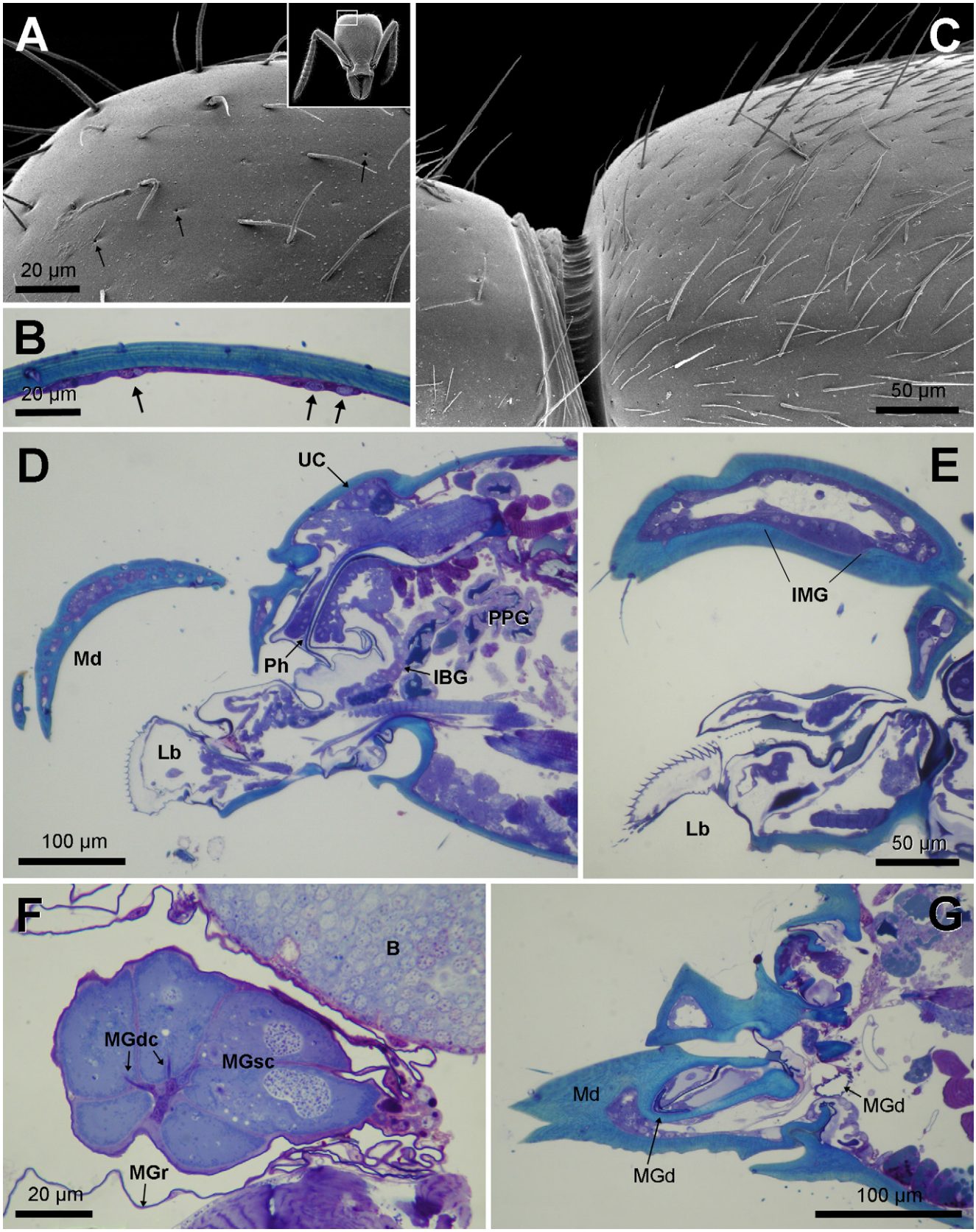
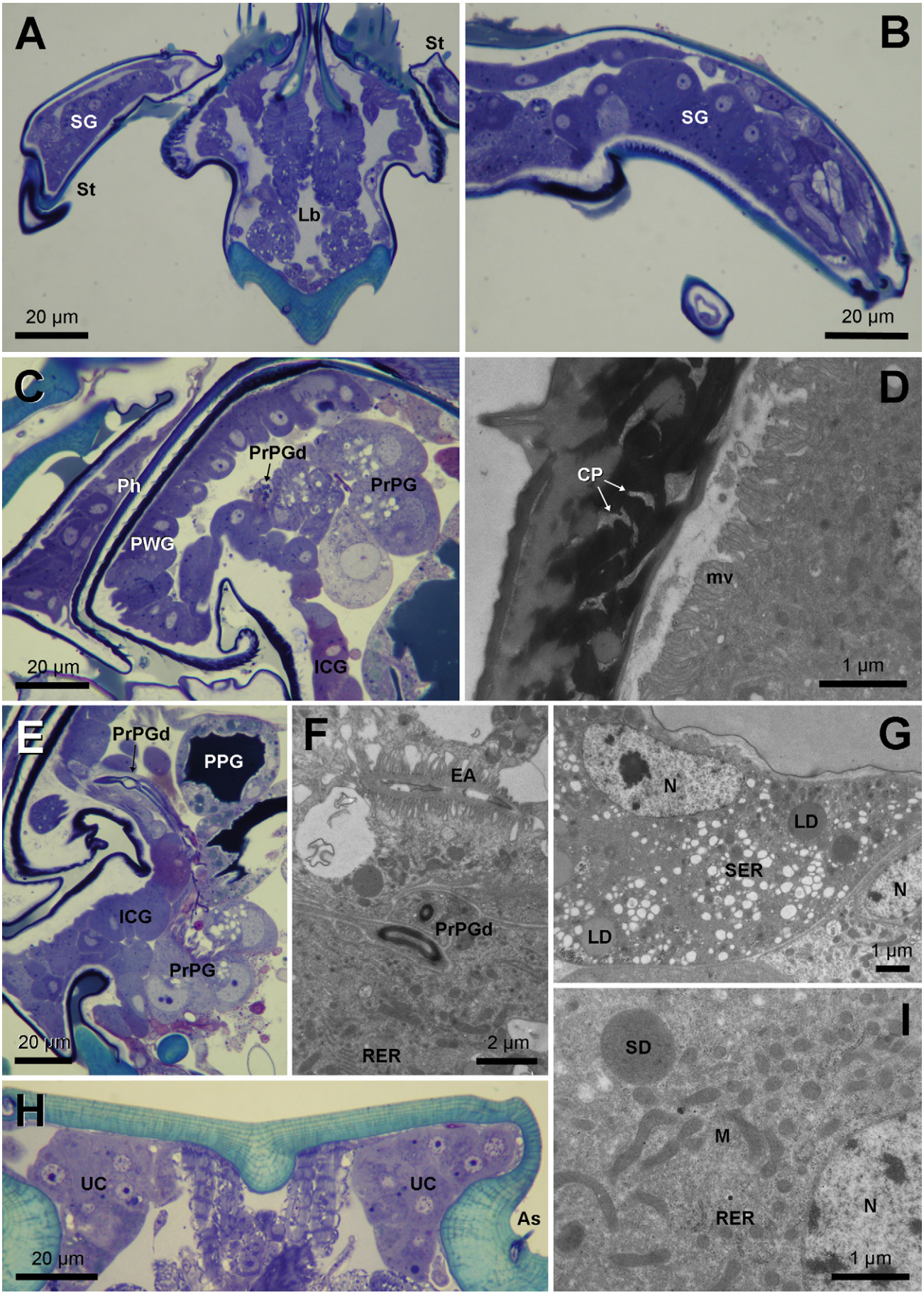


Fig. 2. **A**. Detail of the upper part of a worker head (see inset), showing isolated pores of subepidermal gland cells (arrows). **B**. Cross section through upper part of a queen head, with subepidermal gland cells (arrows) interspersed between epidermal cells. **C**. First gastral tergite of a worker with high concentration of subepidermal gland pores. **D**. Longitudinal section along the midline through the anterior part of a queen's head. **E**. Detail of queen mandible, showing epithelial intramandibular gland in proximal ventral portion. **F**. Cluster of mandibular gland cells and surrounding reservoir (queen). **G**. Longitudinal section through mandibular articulation with the head, showing the T-shaped opening site of the mandibular gland duct in the mandibular base (queen). B: brain, Lb: labium, IBG: infrabuccal cavity gland, IMG: intramandibular gland, Md: mandible, MGd: mandibular gland duct, MGdc: mandibular gland duct cells, MGr: mandibular gland reservoir, MGsc: mandibular gland secretory cells, Ph: pharynx, PPG: postpharyngeal gland, UC: unspecified cell cluster.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case in Leptanillinae (Billen et al., 1998). *P. wallacei* keeps its saber-shaped mandibles opened 180° when defending captured prey (Hölldobler and Wilson, 1990), which probably allows release of mandibular gland substances.

The maxillae contain an **epithelial stipes gland**, which is a novel exocrine structure for social insects. The gland occurs in both workers and queens (Fig. 3A and B) and is formed by an epithelium with a thickness of approx. 20 μm. The epithelium lines the ventral distal part of both stipites. Its function remains unknown, but is likely linked with food processing.

The upper, and especially lower epithelium of the anterior portion of the pharynx in both workers and queens is differentiated into a **pharyngeal wall gland**, of which the lower part continues as an **infrabuccal cavity gland** (Fig. 3C). The glandular epithelium has a thickness of 10–12 μm, with rounded basally located nuclei and a microvillar apical cell border (Fig. 3D). The cuticle covering the glandular area displays conspicuous cuticular pores (Fig. 3D), that will facilitate the discharge of the secretory products. The glandular differentiation of the pharyngeal epithelium represents an exocrine structure that has not yet been reported for social insects; a glandular lining of the infrabuccal cavity was already described in *Monomorium pharaonis* (Eelen et al., 2004). A digestive function appears most likely for both glands because of their association with the pharyngeal tube.

The **propharyngeal gland** is a paired class-3 gland with approx. 6 cells per side in workers and 8 cells per side in queens. The rounded cells have a diameter of 23.7 ± 0.9 μm in workers and 30.4 ± 2.7 μm in queens. The individual ducts of the various gland cells at each side's cluster join into a small ampulla-like space before opening into the lateral pharyngeal wall (Fig. 3E). Ultrastructural observations show the secretory cells with a clear end apparatus and a cytoplasm with granular endoplasmic reticulum (Fig. 3F). The latter is in agreement with the production of digestive enzymes, which is the gland's presumable function (Amaral and Caetano, 2005 – these authors unconventionally called this the 'hypopharyngeal gland' in their work).

More posteriorly occurs the **postpharyngeal gland**, which has the usual glove-shape as in the majority of ants. The various tubular extensions are lined with a monolayered class-1 epithelium (Fig. 3C and E), the thickness of which varies between 5 and 20 μm. The cytoplasm displays an extensive vesicular smooth endoplasmic reticulum (Fig. 3G). This is a common feature of the ants' postpharyngeal gland, the contents of which are hydrocarbons, that have a similar composition as these found on the outer cuticle (Bagnères and Morgan, 1991).

In both workers and queens, we also encountered two conspicuous but as yet **unspecified cell clusters** underneath the frontal part of the head near the antennal insertion (Figs. 2D and 3H). The clusters are each formed by approx. 10 polygonal cells with a diameter of approx. 10 μm and with rounded nuclei of approx. 4 μm. They look like clusters of class-3 gland cells, though we could not find any sign of ducts. Also from the limited material we had available for electron microscopy, we could not get decisive confirmation that the cells have a glandular nature. The cells contain numerous mitochondria and scattered ribosomes, but we could not find an end apparatus (Fig. 3I), which makes these cell clusters a rather puzzling discovery.

3.2. Thoracic glands

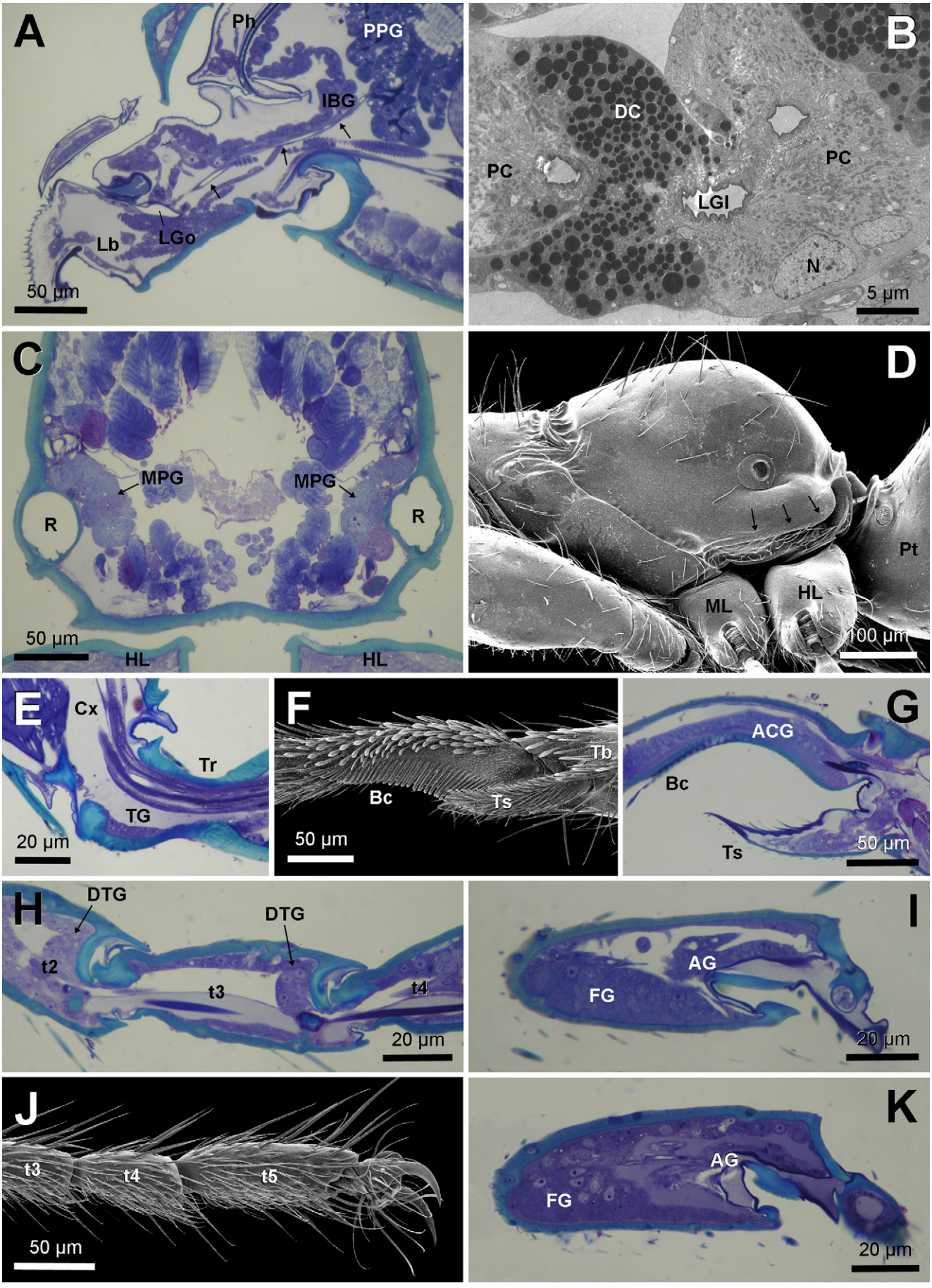
Although opening in the head at the tip of the labium, the secretory part of the **labial** (=**salivary**) **gland** is situated in the thorax. The unpaired part of the cephalic duct, in both workers and queens, forms an ellipsoid dilatation just before opening to the outside (Fig. 4A), which may serve as a small storage chamber for saliva that is 'stand-by' for immediate release. The unpaired duct runs posteriorly through the head, and bifurcates in the region of the neck, both thoracic ducts widening as a reservoir and connecting to the secretory part that is situated in the pro- and mesothorax. The secretory part has a tubular appearance with a diameter around 20 μm, and is formed of class-1 cells lining the central lumen. Two distinctly different cell types occur (Fig. 4B), with dark cells containing an abundance of electron-dense round secretory droplets and a well-developed granular endoplasmic reticulum, and pale cells with numerous mitochondria and loose strands of granular endoplasmic reticulum. The occurrence of granular endoplasmic reticulum is in line with the production of a proteinaceous secretion, as can be expected for the saliva. We did not find any difference between workers and queens, although Hölldobler et al. (1989) described in *Leptanilla japonica* a considerably larger labial gland in the queen. Judging from the micrographs published by these authors, the labial gland in *Leptanilla* appears to be uniformously formed by dark cells only.

The **metapleural gland** is a unique exocrine structure that only occurs in the Formicidae, where it produces antibiotics (Hölldobler and Engel-Siegel, 1984). In *Protanilla*, the paired gland at each side is formed by a cluster of class-3 cells that open into a heavily sclerotized spherical reservoir (Fig. 4C), which in turn opens to the outside through an upward curved and permanently open slit adjacent to the insertion of the hindlegs (Fig. 4D). In workers, 9 cells with a diameter of 27.2 ± 2.6 μm were counted at each side, while queens contained 22 cells per side, with a diameter of 23.3 ± 2.9 μm. Also *Leptanilla* has well developed metapleural glands (Hölldobler et al., 1989), which can be linked with their subterranean hunting lifestyle, that faces them with a variety of microorganisms to be controlled.

Among the thoracic glands are also the exocrine structures that occur in the legs, which in ants can reach the impressive total number of 20 glands (Billen, 2009). Of these, we did encounter the **trochanter gland** in the three leg pairs of both castes (Fig. 4E). This epithelial gland has a thickness of 8 μm and occurs at the ventral proximal portion of the trochanter. It may produce lubricant substances to facilitate the articulation movements with the coxa (Billen, 2009).

The forelegs contain the antenna cleaner apparatus, which is formed by a basitarsal comb and the tibial spur (Fig. 4F). Underneath the basitarsal comb we find a conspicuous epithelium, which in several other ant species has been described as the **antenna cleaner gland** (Schönitzer et al., 1996). The epithelium reaches a thickness of 20 μm. Its anatomical position makes it suggestive to

Fig. 3. A. Cross section through the labium and stipes of a worker, showing the epithelial gland lining the ventral wall of the stipes. **B**. Detail of the stipes of a queen with the conspicuous epithelial gland. **C**. Longitudinal section through the anterior pharynx and the upper part of the infrabuccal cavity of a queen, showing the glandular epithelial lining of both structures. **D**. Electron micrograph detail of the apical region of a worker's pharyngeal wall gland. **E**. Infrabuccal cavity and junction of propharyngeal gland duct cells prior to opening into the lateral pharynx (queen). **F**. Electron micrograph image of propharyngeal gland cytoplasm (worker). **G**. Ultrastructure of a queen's postpharyngeal gland epithelium with vesicular smooth endoplasmatic reticulum. **H**. Cross section through the frontal region of a worker head at the level of the antennal insertions, showing two conspicuous but yet unspecified cell clusters. **I**. Electron micrograph detail of cytoplasm of these unspecified cells (worker). As: antennal socket, CP: cuticular pores, EA: end apparatus, Lb: labium, LD: lipid droplet, ICG: infrabuccal cavity gland, M: mitochondria, mv: microvilli, N: nucleus, Ph: pharynx, PWG: pharyngeal wall gland, PPG: postpharyngeal gland, PrPG: propharyngeal gland, PrPGd: propharyngeal gland duct cells, RER: granular endoplasmic reticulum, SD: secretory droplet, SG: stipes gland, SER: smooth endoplasmic reticulum, St: stipes, UC: unspecified cells.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assign a cleaning function for the antennae to it, although no direct proof is available for such function.

In tarsomeres 2, 3 and 4 of the three leg pairs, we found the **distal tarsomere glands** (Fig. 4H). These appear as a 10 μm thick glandular lining of the invaginated distal cuticle of these tarsomeres. Their position and repetitive occurrence in multiple tarsomeres may support a lubricant function (Billen, 2009).

The pretarsus of the three leg pairs contains the epithelial **arolium gland**, which is the most common of all leg glands, as it is found in the legs of all Hymenoptera, regardless of sex or caste (Billen, 2009). In *Protanilla*, the arolium gland exists but is not particularly conspicuous. It forms the lining of the invaginated distal pretarsal sac and reaches a thickness of 8 μm (Fig. 4I,K).

Besides the arolium gland, we also discovered in the hindleg pretarsus a very pronounced glandular epithelium with a thickness of 15 μm lining the proximal ventral part of the pretarsus (Fig. 4I worker, Fig. 4K queen). A similar epithelium also occurs in the proximal part of the previous tarsomere (t4). The cylindrical cells have basally located rounded nuclei with a diameter of 4 μm. This gland has never been found before in social insects, and therefore represents a novel exocrine structure, that becomes the 21st known gland in the legs of ants (Billen, 2009). Because of its ventral position at the distal end of the leg, we suggest it be called the **foot-sole gland**. It somehow looks as the structural counterpart of the footprint gland that occurs in the proximal dorsal part of the hindleg pretarsi of *Amblyopone* ants (Hölldobler and Palmer, 1989). This footprint gland produces trail pheromones by a peculiar pretarsal twisting during trail deposition (Billen et al., 2005). The ventral location of the novel foot-sole gland of *Protanilla* is well suited for trail-laying as well, although the very hairy appearance of the entire ventral part of the distal tarsomeres (Fig. 4J) may rather compromise proper deposition of substances onto the substrate.

3.3. Abdominal glands

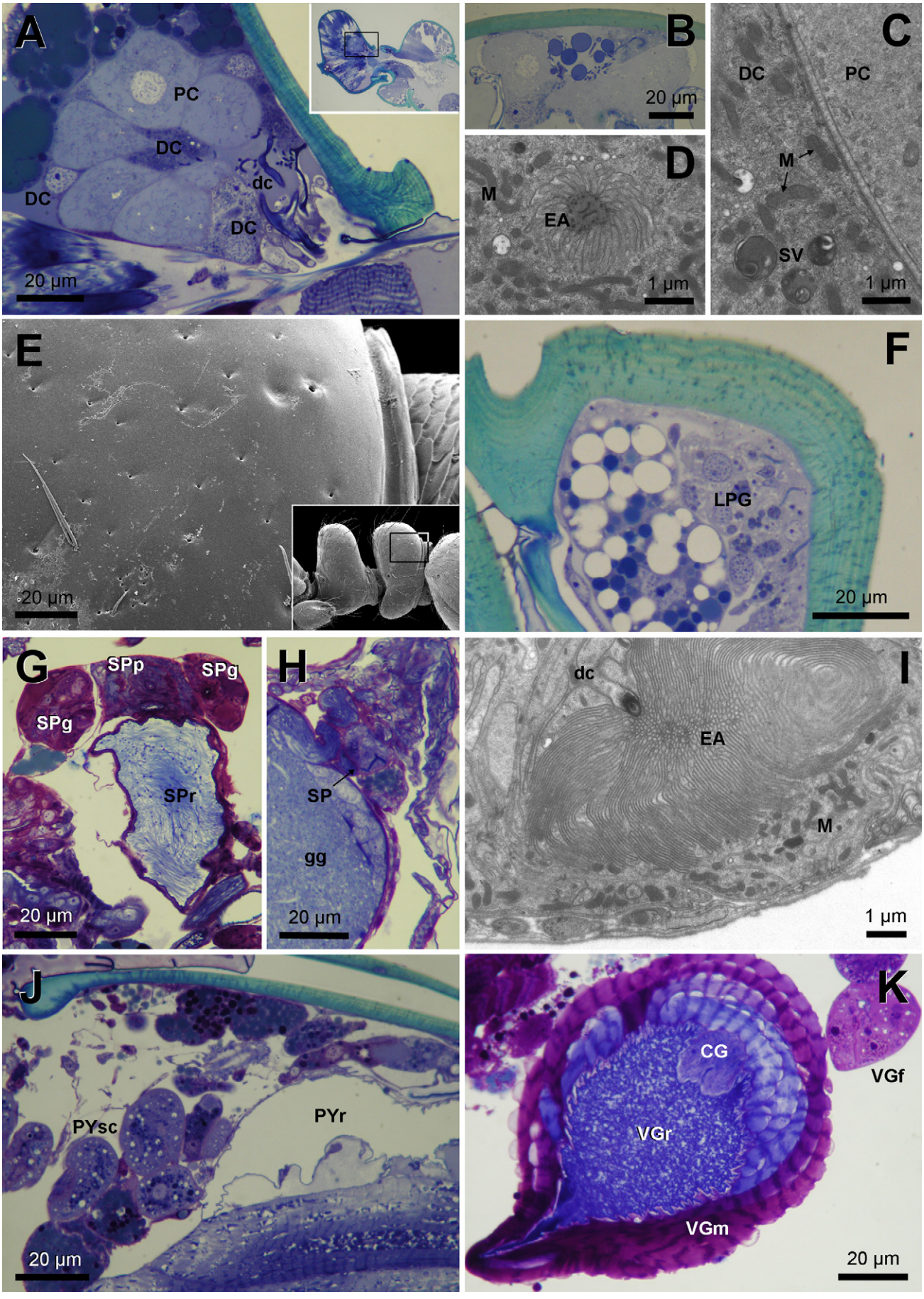
At the posterior side of the petiole, the upper intersegmental membrane that articulates with the postpetiole invaginates anteriorly, and serves as the reservoir space for a prominent unpaired gland, that represents another novel exocrine gland. We suggest to designate this novel structure, that occurs in both castes, as the **central petiole gland**, in order to distinguish it from the isolated class-3 gland cells that open at the lateral petiolar surface. The central petiole gland is formed by a cluster of approx. 20 large rounded cells with a diameter up to 30 μm, with smaller dark cells squeezed between them (Fig. 5A). The invaginated membrane forms a branched system of tubular spaces, as is clear from cross sections (Fig. 5B). Both cell types have rounded nuclei. The dark cells contain numerous mitochondria, dark secretory vesicles, Golgi apparatus and a clear end apparatus, and thus are typical class-3 gland cells (Fig. 5C and D). The more numerous pale cells, however, contain a very uniform cytoplasm without obvious organelles (Fig. 5C). As far as we could check, we could not find an end apparatus, which questions the glandular nature of these cells, in spite of their very apparent clustering together with the darker cells to form an anatomically well-designed unit that is linked through duct cells with the invaginated reservoir space. The precise nature of these large cells therefore remains rather mysterious, while also the function of this novel gland remains unknown.

The lateral surfaces of both the petiole (not shown) and even more so of the postpetiole (Fig. 5E) of both castes display tens of pores with a diameter of 0.5–1 μm, which have the usual appearance and size of duct openings of class-3 cells. They correspond with the presence of isolated subepidermal gland cells underneath, that have been described already by Dejean (1985) in *Smithistruma* ants, but without information about their function. Although not illustrated, postpetiolar gland cells were also reported in a few species of male ants (Hölldobler and Engel-Siegel, 1982). Besides the isolated cells of such (**post**)**petiole glands** in *Protanilla*, we also found a more conspicuous clustered appearance of class-3 gland cells at the dorsolateral region of the postpetiole (Fig. 5F), which has never been described in ants previously. Their obvious concentrated appearance therefore is an argument to consider these paired clusters as a novel **lateral postpetiole gland**. The secretory cells have a diameter of 10–12 μm with round nuclei of 5 μm. Another concentration of presumably gland pores occurs on the dorsolateral surfaces of the first gastral segment (Fig. 2C). The occurrence of a gland in this position would be another novel finding, but as long as no supportive histological data are available, we have to consider the presence of pores as a potential exocrine structure only.

Of all glands reported, the **spermathecal gland** is the only one with significant differences between queen and worker. The spermatheca opens into the oviduct through a spermathecal duct, and further comprises a reservoir, two spermathecal glands and a complex muscular sperm pump. The latter occurs at the junction of the spermathecal duct and the spermathecal glands, and will regulate the number of sperm cells that is released when an egg descends through the oviduct. In the *Protanilla* queens, the two spermathecal glands have a knob-like appearance with a diameter of approx. 20–25 μm (Fig. 5G), whereas in workers the glands are almost degenerated (Fig. 5H, showing the remainder of the sperm pump). The queen glands are formed by class-3 secretory cells that surround the narrow sperm gland duct. The secretory cells are characterized by an end apparatus with extremely long microvilli (Fig. 5I), which is commonly found in ant spermathecal glands (Gobin et al., 2006). Hölldobler et al. (1989) describe a disproportionally large spermatheca in *Leptanilla japonica*, though this seems to apply mainly to the size of the reservoir. In queens of our *Protanilla*, the spermatheca reservoir, in spite of being filled with sperm, is proportionally much smaller than that of the *Leptanilla* queen.

The paired **pygidial gland** in workers and queens consists of ovoid class-3 secretory cells that open through their accompanying ducts into the invaginated intersegmental membrane between the 6th and 7th tergite (Fig. 5J). In *Leptanilla*, no pygidial gland could be found (Hölldobler et al., 1989). On the other hand, the *Leptanilla japonica* queen displays an impressive multitude of large intersegmental tergal and sternal glands (Hölldobler et al., 1989), of which we did not find any trace in *Protanilla*. The presence of these

Fig. 4. **A**. Longitudinal section through the anterior tip of the head and labium of a worker at the region of labial gland opening. Note the ampulla-like widening of the labial gland duct just before it opens to the outside. Arrows indicate labial gland duct. **B**. Electron micrograph along labial gland tubule (queen), clearly showing the presence of dark and pale cells. **C**. Cross section through the metathorax of a queen, showing the metapleural glands. **D**. Scanning micrograph of the posterior thorax of a worker, with arrows indicating the curved slit-like opening of the metapleural gland. **E**. Longitudinal section through the coxa–trochanter junction in a worker hindleg with location of the trochanter gland. **F**. Scanning micrograph of the antenna cleaner apparatus in a worker foreleg. **G**. Longitudinal section through the antenna cleaner apparatus in a worker foreleg, showing the presence of the antenna cleaner gland. **H**. Longitudinal section through tarsomeres 2–4 of a queen's midleg with indication of the repetitive distal tarsomere glands. **I**. Longitudinal section through the hindleg pretarsus (worker) showing the arolium gland and the foot-sole gland. **J**. Scanning micrograph through the distal tarsomeres of a worker hindleg, showing the uniformly hairy appearance. **K**. Longitudinal section through the hindleg pretarsus of a queen. ACG: antenna cleaner gland, AG: arolium gland, Bc: basitarsal comb, Cx: coxa, DC: dark cells, DTG: distal tarsomere gland, FG: foot-sole gland, HL: hindleg, IBG: infrabuccal cavity gland, Lb: labium, LGI: labial gland lumen, LGo: labial gland opening, ML: midleg, MPG: metapleural gland, N: nucleus, PC: pale cells, Ph: pharynx, PPG: postpharyngeal gland, Pt: petiole, R: reservoir, t2–5: tarsomeres 2–5; Tb: tibia, TG: trochanter gland, Tr: trochanter, Ts: tibial spur.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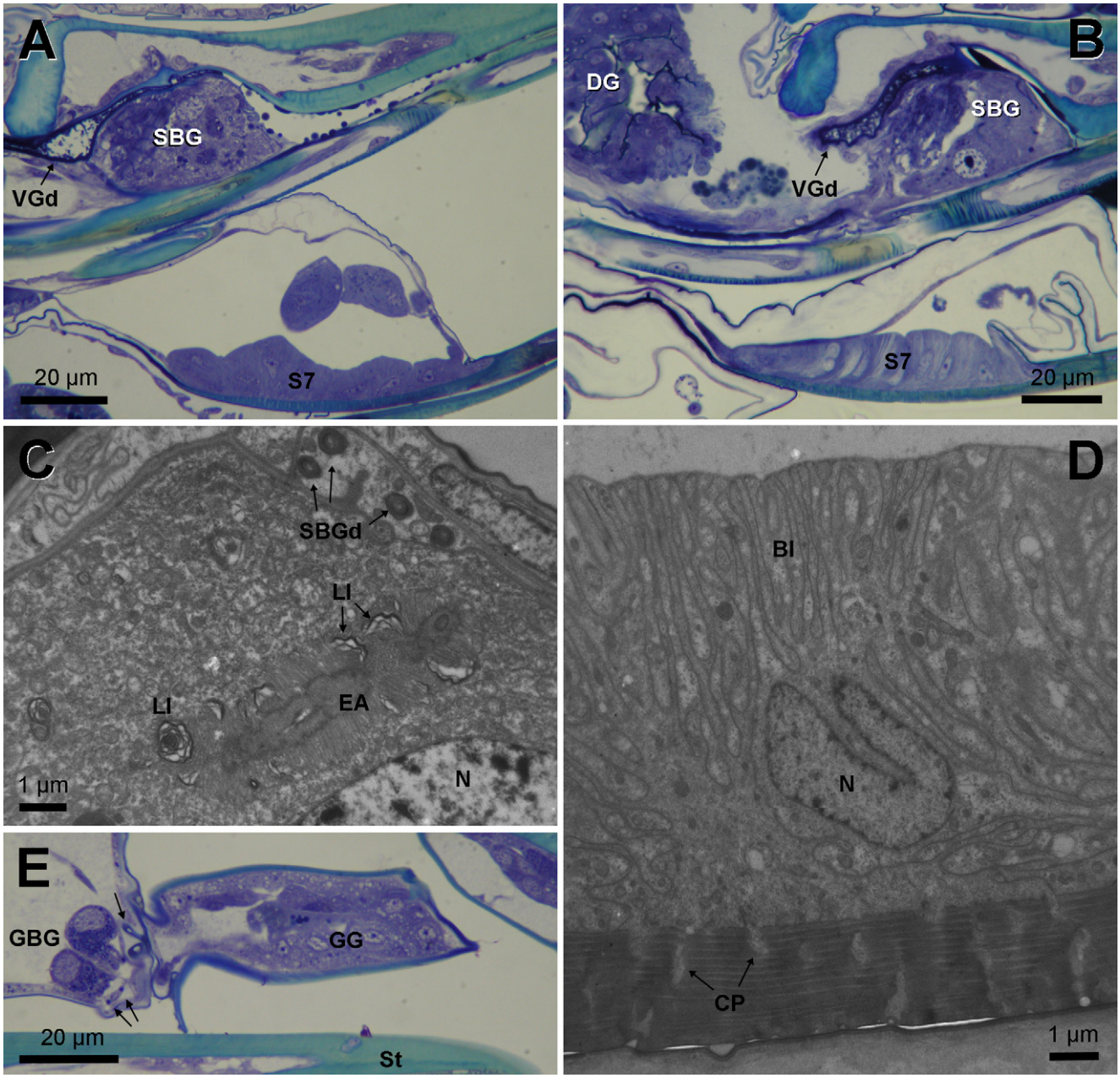

Fig. 6. **A**. Longitudinal section through the sting base and anterior portion of the 7th sternite in a worker. **B**. Same in queen. **C**. Electron micrograph of a sting bulb gland cell of a queen. **D**. Ultrastructure of the epithelium of the 7th sternite gland, showing conspicuous basal invaginations and cuticular pores. **E**. Longitudinal section through a gonostylus, showing both the internal gonostyli gland and the gonostyli base gland (arrows indicate duct cells). BI: basal invaginations, CP: cuticular pores, DG: Dufour gland, EA: end apparatus, GBG: gonostyli base gland, GG: gonostyli gland, LI: lamellar inclusion, N: nucleus, S7: 7th sternite epithelial gland, SBG: sting bulb gland, SBGd: sting bulb gland ducts, St: sting, VGd: venom gland duct.

intersegmental gland complexes may be related to physogastry of the *Leptanilla* queen, as such intersegmental glands are lacking in *Leptanilla* workers (Hölldobler et al., 1989) as well as in the ergatoid queens of *Protanilla*.

The **Dufour gland** in *Protanilla* is rather small and bulbous, and opens through the sting base. Due to the folded appearance of the epithelium, an irregular epithelial thickness between 4 and 13 µm was measured (Fig. 6B).

Fig. 5. **A**. Longitudinal section through the central petiole gland in a queen (area indicated in inset). **B**. Cross section through the region of central petiole gland (worker) where ducts open into the branched tubiform reservoir space. **C**. Electron micrograph of the cytoplasm of the two cell types of the central petiole gland (queen). **D**. Detail of an end apparatus in the dark cell type of the central petiole gland (queen). **E**. Lateral surface of a queen postpetiole (area indicated in inset) showing abundance of pores. **F**. Longitudinal section through the lateral postpetiole gland, showing ducts opening through the cuticle. **G**. Cross section of queen spermatheca with sperm-filled reservoir and spermathecal glands. **H**. Cross section of atrophied spermatheca in a worker. **I**. Detail of an end apparatus in the queen spermathecal gland with very long microvilli. **J**. Longitudinal section through the 7th tergite and underlying pygidial gland in a worker. **K**. Worker's venom gland, showing very well-developed muscular surrounding of the reservoir. CG: convoluted gland, dc: duct cells, DC: dark cells, EA: end apparatus, gg: ganglion, LPG: lateral postpetiole gland, M: mitochondria, PC: pale cells, PYr: pygidial gland reservoir, PYsc: pygidial gland secretory cells, SP: spermatheca, SPg: spermathecal gland, SPp: sperm pump, SPr: spermathecal reservoir, SV: secretory vesicle, VGf: venom gland filament, VGm: venom gland musculature, VGr: venom gland reservoir.

Fig. 7. Trail-following at the onset of nest migration in *Protanilla wallacei*. Note the participation of both workers and queens (Q), with two of the queens helping in transport of larvae. The trail party is shown when leaving through the entrance tunnel of their artificial nest. NW: nest wall.

The **venom gland** consists of the usual components, being two slender secretory filaments, a convoluted gland and a reservoir, that tapers into a narrow duct that will open through the sting base. The secretory filaments have a diameter of 20–25 μm and contain class-3 glandular cells that are arranged around the central filament lumen. The most striking characteristic of the gland is the extremely thick muscular surrounding of the reservoir (Fig. 5K). Hölldobler et al. (1989) already described an unusually large venom gland in *Leptanilla*, also with a massive muscular surrounding of the reservoir, which was reported as the most muscular venom gland known for ants. Compared with *Leptanilla*, however, the muscular supply of *Protanilla* is even far more developed, therefore making them the ants with the most muscular venom gland. This exceptional development of the venom gland in *Protanilla* is probably in agreement with their feeding on relative large dipluran prey items, that need to be paralysed by powerful venom injection through the sting.

A very unexpected finding was the presence of a **sting bulb gland** in both workers (Fig. 6A) and queens (Fig. 6B). The gland occurs as a cluster of approx. 5–6 class-3 glandular cells that is situated inside the base of the sting bulb. The glandular cells have a diameter of 15–18 μm. They have a clear end apparatus, with secretion that appears as lamellar inclusions, found between the microvilli of the end apparatus, that are on their way to the duct cell (Fig. 6C). A sting bulb gland, with the same characteristics as described here, so far has only been reported for the endemic Australian genera *Myrmecia* and *Nothomyrmecia* (Billen, 1990). This makes its presence in *Protanilla* very surprising and puzzling, given its different and distant phylogenetic position from the Myrme-ciinae.

Under the anterior side of the last sternite, an unpaired **7th sternite gland** is found in both castes (Fig. 6A and B). The epithelium has an average thickness of 12–15 μm. The cylindrical cells display very extensive basal invaginations that reach up to half the cells' height, while the cuticle shows conspicuous pores, that facilitate the discharge of the secretory products to the outside (Fig. 6D). A similar large median gland underneath the 7th sternite was also reported by Hölldobler et al. (1989) in workers of *Leptanilla japonica*, though it is absent in the queen. The observation of *L. japonica* workers lowering their abdomens to touch the substrate during trail-laying (Masuko, 1990) suggests the involvement of a sternal gland in this behaviour, for which the 7th sternite gland is a likely candidate (Hölldobler et al., 1989). Trail-laying may also be a possible function for *Protanilla* (see further).

We also found two glands associated with the gonostyli. At the base of the gonostyli, we observed a small cluster of 3–4 class-3 gland cells of the **gonostyli base gland**. The round cells have a diameter of approx. 10 μm, and look dark and granular as they appear loaded with secretory inclusions (Fig. 6E). A gonostyli base gland has also been described in some ponerine genera (*Diacamma*, *Odontomachus*, *Harpegnathos* and *Leptogenys*) by Jessen et al. (1979) and Jessen and Maschwitz (1983), although in these cases the gland was formed by numerous gland cells while the duct cells were also much longer. The similar anatomical position in *Protanilla* at the gonostylar base, however, indicates it is the same glandular structure. Inside the gonostyli, we found the **gonostyli gland**, that appears as a layer of class-1 epithelial cells lining the ventral cuticle (Fig. 6E). A similar glandular epithelium was also found in the gonostyli of *Diacamma* sp. (Jessen et al., 1979) and *Pachycondyla tridentata* (Jessen and Maschwitz, 1983), with a class-3 gonostylar gland occurring as well in these species.

4. Conclusion

Our survey revealed the presence of 26 glands in *P. wallacei*, including the unexpected discovery of six glands that had never been reported before in ants, five of these being novel findings for social insects as such. Besides these many glands, we also encountered another two potential exocrine structures: a so far unspecified pair of cell clusters that occur frontally in the head, and the appearance of numerous pores on the dorsolateral part of the first gastral tergite, that may correspond with gland cells underneath.

We found a high degree of similarity between the glands of workers and queens, with only a clear difference in the spermathecal gland, that is well developed in queens while very reduced in workers. This is in contrast with *Leptanilla*, where Hölldobler et al. (1989) found significant caste differences for several glands. While the monogynous *Leptanilla* are characterized by a pronounced caste dimorphism, our *P. wallacei* colony has multiple ergatoid queens, that are also behaviourally more similar to workers. These worker-like traits are in line with the similarities in the exocrine system of both castes. We observed queens actively participating during the event of nest migration, following the trail and assisting in carrying larvae (Fig. 7). Trail laying may be associated with the epithelial gland on the 7th sternite, as was suggested for *Leptanilla* by Hölldobler et al. (1989). The presence of this gland in workers and

queens of *Protanilla*, but in workers only of *Leptanilla*, could be understood in this regard. On the other hand, also the newly described foot-sole gland of *Protanilla* occurs in an anatomical position that is very suitable for trail laying, and is indeed found in both workers and queens. The presence of long hairs on the external surface of the gland region (Fig. 4J), however, may be an argument against a function in trail deposition. To find out about the real origin of the trail substance, and about the function of several other glands, will remain a challenging task whenever live ants of this fascinating species may become available again in future.

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References

Amaral, J.B., Caetano, F.H., 2005. The hypopharyngeal gland of leaf-cutting ants (*Atta sexdens rubropilosa*) (Hymenoptera: Formicidae). Sociobiology 46, 515–524.

Bagnères, A.G., Morgan, E.D., 1991. The postpharyngeal gland and the cuticle of Formicidae contain the same characteristic hydrocarbons. Experientia 47, 106–111.

Billen, J., Ito, F., Maile, R., Morgan, E.D., 1998. The mandibular gland, probably the source of the alarm substance in *Leptanilla* sp. (Hymenoptera, Formicidae). Naturwissenschaften 85, 596–597.

Billen, J., Thys, B., Ito, F., Gobin, B., 2005. The pretarsal footprint gland of the ant *Amblyopone reclinata* (Hymenoptera, Formicidae) and its role in nestmate recruitment. Arthropod Structure and Development 34, 111–116.

Billen, J., 1990. The sting bulb gland in *Myrmecia* and *Nothomyrmecia* (Hymenoptera: Formicidae): a new exocrine gland in ants. International Journal of Insect Morphology and Embryology 19, 133–139.

Billen, J., 2009. Occurrence and structural organization of the exocrine glands in the legs of ants. Arthropod Structure and Development 38, 2–15.

Bolton, B., 1990. The higher classification of the ant subfamily Leptanillinae (Hymenoptera: Formicidae). Systematic Entomology 15, 267–282.

Bolton, B., 1995. A New General Catalogue of the Ants of the World. Harvard University Press, Cambridge, Mass., London.

Costa Leonardo, A.M., 1978. Glândulas intramandibulares em abelhas sociais. Ciência e Cultura 30, 835–838.

Dejean, A., 1985. Etude eco-éthologique de la predation chez les fourmis du genre *Smithistruma* (Formicidae-Myrmicinae – Dacetini). II. Attraction des proies principales (Collemboles). Insectes Sociaux 32, 158–172.

Eelen, D., Børgesen, L.W., Billen, J., 2004. Morphology of a novel glandular epithelium lining the infrabuccal cavity in the ant *Monomorium pharaonis* (Hymenoptera, Formicidae). Arthropod Structure and Development 33, 471–475.

Gobin, B., Ito, F., Billen, J., 2003. The subepithelial gland in ants: a novel exocrine gland closely associated with the cuticle surface. Acta Zoologica (Stockholm) 84, 285–291.

Gobin, B., Ito, F., Peeters, C., Billen, J., 2006. Queen-worker dimorphism in the spermathecae of phylogenetically primitive ants. Cell Tissue Research 326, 169–178.

Grasso, D.A., Romani, R., Castracani, C., Visicchio, R., Mori, A., Isidoro, N., Le Moli, F., 2004. Mandible associated glands in queens of the slave-making ant *Polyergus rufescens* (Hymenoptera, Formicidae). Insectes Sociaux 51, 74–80.

Hölldobler, B., Engel-Siegel, H., 1982. Tergal and sternal glands of male ants. Psyche 89, 113–132.

Hölldobler, B., Engel-Siegel, H., 1984. On the metapleural gland of ants. Psyche 91, 201–224.

Hölldobler, B., Palmer, J.M., 1989. Footprint glands in *Amblyopone australis* (Formicidae, Ponerinae). Psyche 96, 111–121.

Hölldobler, B., Wilson, E.O., 1990. The Ants. Harvard University Press, Cambridge, Mass.

Hölldobler, B., Palmer, J.M., Masuko, K., Brown Jr., W.L., 1989. New exocrine glands in the legionary ants of the genus *Leptanilla* (Hymenoptera, Formicidae, Leptanillinae). Zoomorphology 108, 255–261.

Jessen, K., Maschwitz, U., 1983. Abdominaldrüsen bei *Pachycondyla tridentata* (Smith): Formicidae, Ponerinae. Insectes Sociaux 30, 123–133.

Jessen, K., Maschwitz, U., Hahn, M., 1979. Neue Abdominaldrüsen bei Ameisen. I. Ponerini (Formicidae, Ponerinae). Zoomorphologie 94, 49–66.

Kück, P., Garcia, F.H., Misof, B., Meusemann, K., 2011. Improved phylogenetic analyses corroborate a plausible position of *Martialis heureka* in the ant tree of life. PLoS ONE 6, e21031.

Masuko, K., 1989. Larval hemolymph feeding in the ant *Leptanilla japonica* by use of a specialized duct organ, the "larval hemolymph tap" (Hymenoptera: Formicidae). Behavioural Ecology and Sociobiology 24, 127–132.

Masuko, K., 1990. Behavior and ecology of the enigmatic ant *Leptanilla japonica* Baroni Urbani (Hymenoptera: Formicidae: Leptanillinae). Insectes Sociaux 37, 31–57.

Moreau, C.S., Bell, C.D., Vila, R., Archibald, S.B., Pierce, N.E., 2006. Phylogeny of ants: diversification in the age of the angiosperms. Science 312, 101–104.

Noirot, C., Quennedey, A., 1974. Fine structure of insect epidermal glands. Annual Review of Entomology 19, 61–80.

Schoeters, E., Billen, J., 1994. The intramandibular gland, a novel exocrine structure in ants (Insecta, Hymenoptera). Zoomorphology 114, 125–131.

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

Ward, P.S., 2007. Phylogeny, classification, and species-level taxonomy of ants (Hymenoptera: Formicidae). Zootaxa 1668, 549–563.

Wheeler, G.C., Wheeler, J., 1988. The larva of *Leptanilla japonica*, with notes on the genus (Hymenoptera: Formicidae: Leptanillinae). Psyche 95, 185–189.