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The pretarsal footprint gland of the ant *Amblyopone reclinata* (Hymenoptera, Formicidae) and its role in nestmate recruitment

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Abstract

Workers of the ant *Amblyopone reclinata* employ solitary prey retrieval when prey is small, but recruit nestmates to large prey. In the latter case, the scout forager paralyses the prey with its powerful sting, and quickly returns to the nest. During this homeward journey, it deposits a trail pheromone, that originates from the well developed footprint glands in its hindlegs. Recruited workers follow this trail to reach the prey, which is then jointly dragged to the nest. The footprint gland is only found in ants of the genus *Amblyopone*, and is formed by a glandular differentiation of the dorsal tegumental epidermis in the hindleg pretarsi. The secretory epithelium is approximately 15–20 μm thick, and shows apical microvilli and basal invaginations. The cytoplasm contains numerous mitochondria. Narrow pores with a diameter of 0.1 μm run through the cuticle, although they were not seen to open at the pretarsus external surface. Careful observation of trail-laying workers reveals that during trail-laying the hindleg pretarsus is twisted in a peculiar position, which explains how secretion from the dorsally located footprint gland is deposited onto the substrate.

Keywords: Morphology; Ultrastructure; Footprint gland; Pretarsus; Recruitment; Ants; *Amblyopone reclinata*

1. Introduction

Cooperation between workers is characteristic for social insect colonies in general, and for ants in particular. A typical example is provided by joint retrieval of large prey. During such group retrieval the scout worker that initially discovered prey, returns to the nest and communicates the location of this discovery to its nestmates. During the recruitment process, chemical trails which lead recruited workers to the food source are often involved (Hölldobler and Wilson, 1990).

On the other hand, in several so-called primitive ants, only solitary foraging and prey retrieval are known (Abe and Uezu, 1977; Maschwitz et al., 1979). Ant species of the genus *Amblyopone* have for long been considered to be solitary hunters that do not employ recruitment of nestmates to prey (Masuko, 1993; Traniello, 1978). However, data indicating that group recruitment can occur in that genus, too, were reported by Hölldobler and Palmer (1989a) for *Amblyopone australis* and by Ito (1993a) for *Amblyopone reclinata*. The latter species has small colonies with an average number of 96 ± 51 workers (Ito, 1993b). In the present study, we further explored the process of prey retrieval in this species in order to find out how recruitment of nestmates is organized.

As will be shown in the present paper, the footprint gland plays a specific role in nestmate recruitment in *Amblyopone*. This epithelial gland is situated in the dorsal part of the hindleg pretarsi and was first reported by Hölldobler and Palmer (1989a) for *Amblyopone australis*. The paper provided a general histological description of this gland, together with a brief mentioning that pilot tests suggested that footprint secretions elicit some unprecise trail following. The present investigation on *A. reclinata* illustrates a very clear trail following activity in this species, and gives structural details that differ from the histological description in *A. australis*.

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2. Material and methods

Three colonies of *A. reclinata* (Mayr, 1879) were collected in the Bogor Botanical Gardens, Indonesia, and were transferred to artificial nests in the laboratory (respective colony size 82, 39 and 35 workers). For two colonies, these nests consisted of a plastic box of $20\times20\times5\ cm^3$ with plaster floor. Within that box, a small glass covered compartment ($8\times5\ cm^2$) served as the actual nest. From here, the ants could enter the foraging arena, where prey was offered. The third colony was kept in the same kind of nest, but had a larger foraging arena ($60\times30\ cm^2$), which allowed us to perform bridge choice experiments (see further). Although the diet of *A. reclinata* in nature generally consists of centipedes (Masuko, 1993), these ants can conveniently be raised on a diet of *Tenebrio molitor* larvae. In our experiments, every other day a mealworm of well-known length and weight was offered in the foraging arena, after which the behaviour of the ants was observed until the prey was transported into the nest.

2.1. Behavioural observations

(a) *Trail marking*. In order to check for the eventual marking of the trajectory between the prey site and the nest entrance, a foraging worker was released on a platform on which a live mealworm was offered. This platform was connected to the foraging arena via two almost parallel bridges (Fig. 1). The floor of each bridge contained a strip of paper, which could be replaced by the strip taken from the other bridge after the return of the scout to the nest but prior to the departure of the recruited nestmates to the prey.

(b) *Source of trail signals*. Determination of the anatomical origin of the trail substance was done by presenting circular trails with a hexane extract of various glands to foragers, and by the analysis of the distance followed by the ants, as detailed in Pasteels and Verhaeghe (1974). Each of the various extracts was tested three times, each time with 10 ants in the testing arena.

(c) *Trail pattern*. In order to visualise the trail pattern, normal walking (i.e. prior to contact with prey; $n=20$) and trail-laying workers (i.e. returning to the nest after prey finding; $n=20$) were forced to walk over a strip of sooted glass, which results in a clearly visible footprint pattern. Observation of the leg position was achieved via filming walking ants in profile with a Sony Handycam DCR-TRV70K.

2.2. Morphology and ultrastructure

The distal parts of the tarsus of fore-, mid- and hindlegs were fixed in cold 2% glutaraldehyde, buffered at pH 7.3 with 50 mM Na-cacodylate and 150 mM saccharose. Postfixation was carried out in 2% osmium tetroxide in the same buffer. Tissues were dehydrated in a graded acetone series, embedded in Araldite and sectioned with a Reichert Ultracut E microtome. Semithin 1 μm sections were stained with methylene blue and thionin and viewed in a Zeiss Axioskop microscope, double stained 70 nm thin sections were examined in a Zeiss EM900 electron microscope.

3. Results

3.1. Behavioural observations

When *A. reclinata* scouts discover a prey, they quickly attack it and, during a brief struggle, sting it until paralysis

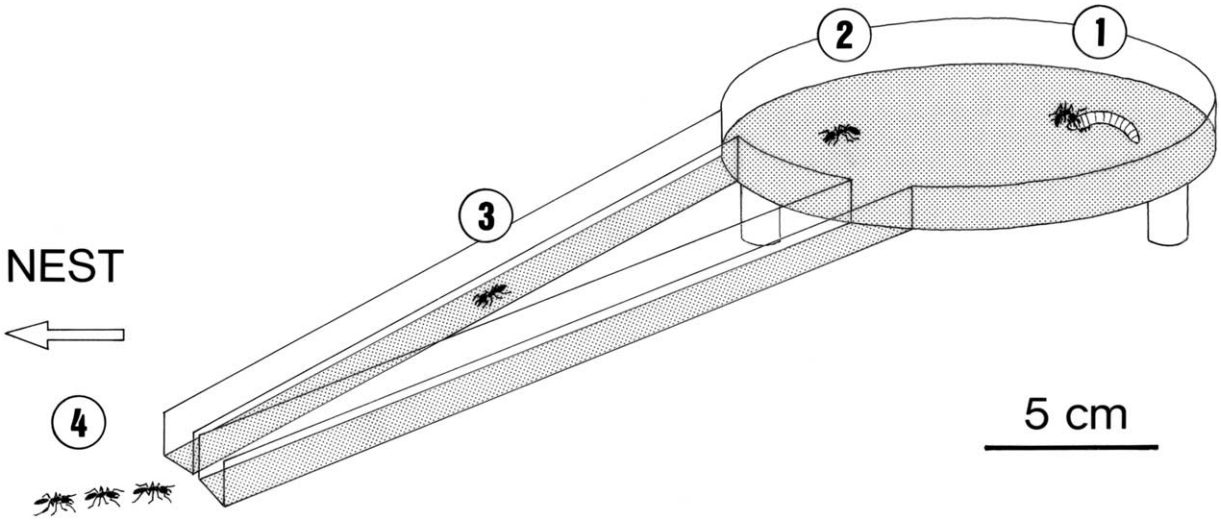

Fig. 1. Schematical set-up of the bridge choice experiment. After finding and paralysing a large mealworm on the platform (1), the scout ant has to return to the nest via one of the two bridges (2,3). Before recruited nestmates depart towards the prey, the floor strips of both bridges may either be left untouched, or exchanged to test the eventual presence of orientation cues for the recruited nestmates on the substrate (4).

sets in (Fig. 2A). When the prey is small, the scout will carry it to the nest alone (Fig. 2B). However, when the prey is large, the worker does not attempt to carry it, but quickly returns to the nest. Immediately after the scout's arrival, several nestmates leave the nest and walk towards the paralysed prey. Together, they will then drag the prey to the nest (Fig. 2C). The number of recruited nestmates is proportional to the size of the prey (Gobin et al., submitted).

The recruited nestmates clearly follow the trajectory used by the inbound scout, which suggests the presence of trail marking. We could experimentally demonstrate the use of trail communication by means of a double bridge system in which we could exchange part of the substrate (Fig. 1). This happened immediately after a recruiting scout (i.e. after its subduing of a large mealworm) had walked over one of the bridges. In 10 trials in which we had exchanged the substrate, 151 out of 156 ants chose the bridge that had the trail on it. Using hexane extracts of various candidate glands or body parts in a circular trail test, we were able to determine that it was the hindleg pretarsus extract that elicited active trail following (Fig. 3). Since, the hindleg pretarsus contains both the arolium gland and the dorsal footprint gland epithelium (see further), we refined the trail test by splitting the pretarsus with a sharp razorblade in an upper and lower part, and tested the extracts of both these parts separately. These tests confirmed that the dorsal part of the pretarsus (containing the footprint gland epithelium) was the source of the trail pheromone (Fig. 3). The slight activity of the extract of the ventral part of the pretarsus (containing the arolium gland) was probably due to its contamination by a small part of the dorsal epithelium, as it was very hard to perfectly split the upper and lower parts of the pretarsus.

The discovery of the dorsal footprint gland as the likely source of the trail pheromone was puzzling, as its secretory products have no direct access to the substrate. Careful analysis of the walking behaviour of foragers and returning scouts revealed that scout ants have a peculiar gait. During trail laying, they twist their hindleg pretarsus in such a way that the dorsal part briefly touches the substrate, and then they are rolling the pretarsus back into its normal position (Fig. 4A). Trail deposition, therefore, occurs during this peculiar stepping behaviour rather than during leg dragging. This observation was confirmed by the distinct pointed trail marks that both normally walking foragers and returning scouts leave on sooted glass (Fig. 4B and C).

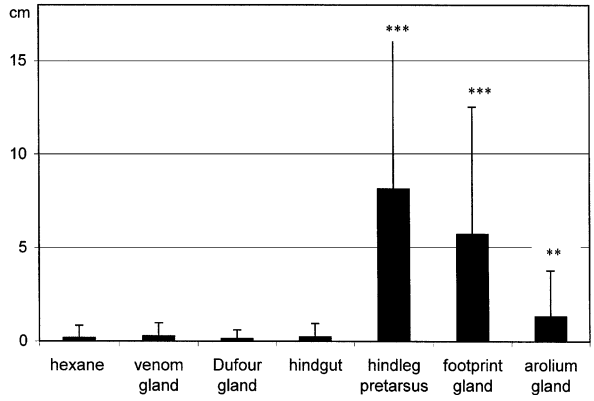

Fig. 3. Trail following activity of workers expressed as mean distance walked (in cm) along a circular trail of hexane and several gland extracts. Asterisks indicate a significant difference from hexane (GLM ANOVA and Tukey HSD post hoc test ***$p=0.002$; **$p=0.05$).

3.2. Morphology and ultrastructure

The pretarsus is the most distal segment of the leg, to which the arolium and two claws articulate. The epidermis underneath the pretarsal cuticle generally occurs as a very flat epithelium with a thickness of hardly 2 μm. A transverse ventral slit forms the opening site of the pretarsal arolium gland, which is formed by a glandular differentiation of the infolded pretarsal epithelium (Fig. 5A). This arolium gland is present in all the the six legs, and is a common gland in the legs of all Hymenoptera (Billen, unpubl. obs.). The hindlegs of *A. reclinata* (as well as *A. australis*: Hölldobler and Palmer, 1989a) contain an additional glandular tissue, as the dorsal epidermis occurs as a considerably thickened epithelium (Fig. 5A and B). This thick epithelium does not occur in the fore- and midlegs, and so far has never been found in other ant species.

The cylindrical epithelial cells have a height of 15–20 μm and a width of 5–10 μm, with centrally located rounded nuclei (Fig. 6A). The overlaying pretarsal cuticle is

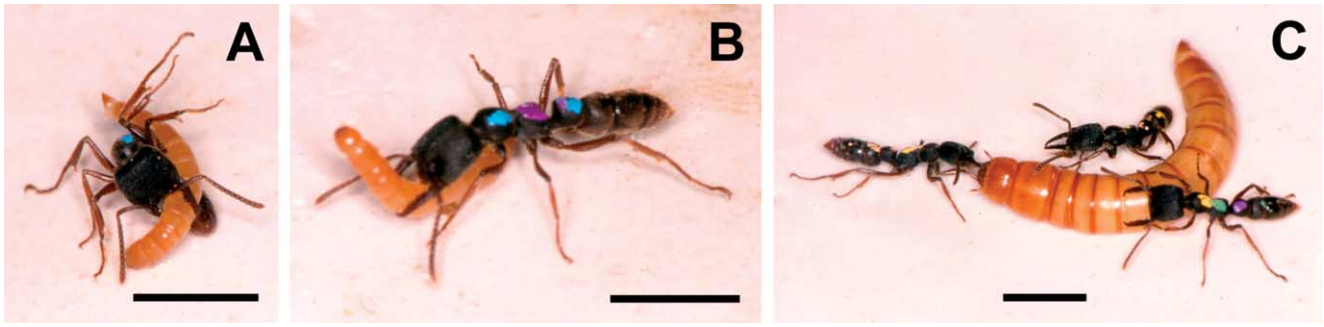

Fig. 2. A. Worker of *A. reclinata* stinging a small mealworm. B. A paralysed small mealworm is carried to the nest by a single worker. C. A large mealworm is carried to the nest by several workers (scale bars 0.5 cm).

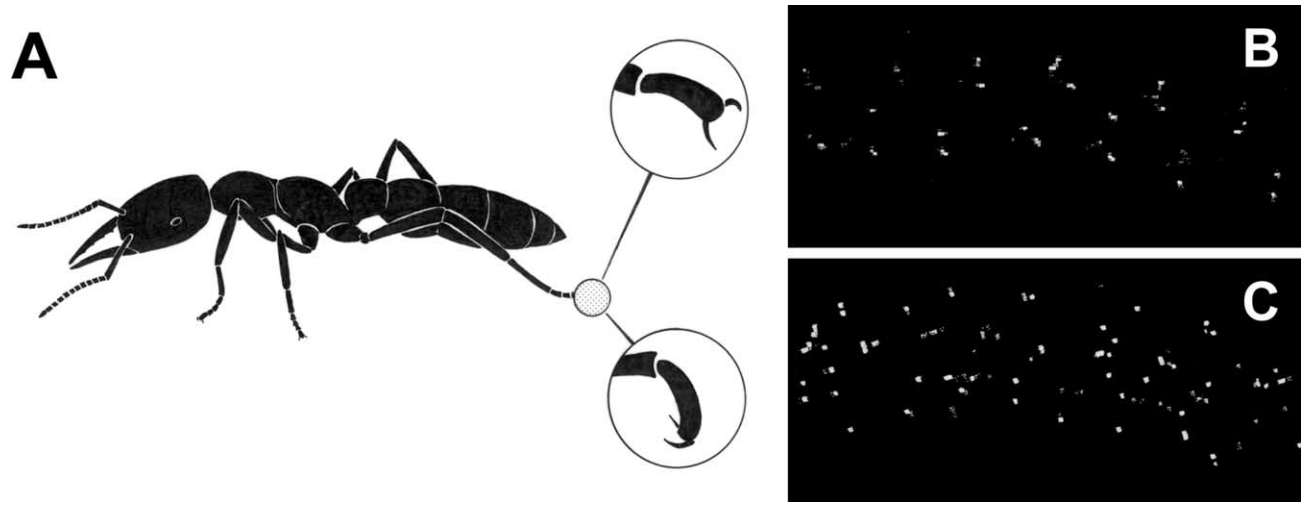

Fig. 4. Schematical representation of the position of the hindleg pretarsus during normal walking and during trail laying (A). In the latter situation, each step of the hindleg is characterized by a downward movement of the pretarsus in a twisted position, allowing the surface of the footprint gland cuticle to touch the substrate. (B) shows the pattern of discontinuous leg marks left by a normally walking worker on a sooted glass, (C) are equally discontinuous marks left by a trail-laying scout worker, which correspond to the peculiar way of trail deposition.

approximately 10 μm thick, and shows a small number of narrow pores with a diameter of 0.1 μm that run perpendicularly to the cuticular surface. The pores start their trajectory from the inner border of the cuticular wall (Fig. 6B), and they could be followed to just underneath the outer epicuticular layer. Sections through pore openings at the surface were, however, never seen, and scanning microscopy of the pretarsal surface also did not reveal any pores. The apical cell membrane is differentiated into short and irregular microvilli (Fig. 6B). The cytoplasm is characterized by an abundance of mitochondria and free ribosomes, while the presence of other organelles was not obvious (Fig. 6A and C). The basal cell membrane shows many conspicuous invaginations, that allow tracheoles to penetrate (Fig. 6D).

4. Discussion

Confrontation with large prey leads to recruitment of nestmate workers in the ant *A. reclinata* (Ito, 1993a). We could experimentally demonstrate the use of trail communication to recruit nestmates by means of a substrate switching experiment. Presentation of extracts of several glands allowed the identification of the footprint gland as the source of the trail pheromone. This gland is situated on the dorsal side of the hindleg pretarsi. The use of trail pheromones originating from exocrine glands in the hindlegs is not exceptional in ants. A common feature in these glands is that the gland opens towards the substrate, which allows efficient deposition of the trail substances. Workers of the genus *Crematogaster* are known to lay trail pheromones from a tibial gland in their hindlegs, which releases its secretion at the tip of the pretarsus (Leuthold, 1968). *Onychomyrmex* and *Prionopelta* workers use a basitarsal gland for this purpose, in which the deposition of the pheromonal substances onto the substrate is achieved through a peculiar behaviour of walking with stiffly outstretched hindlegs in order to bring the gland opening in contact with the substrate (Hölldobler and Palmer, 1989b; Hölldobler et al., 1992). In the first description of the footprint gland in *A. australis*, Hölldobler and Palmer

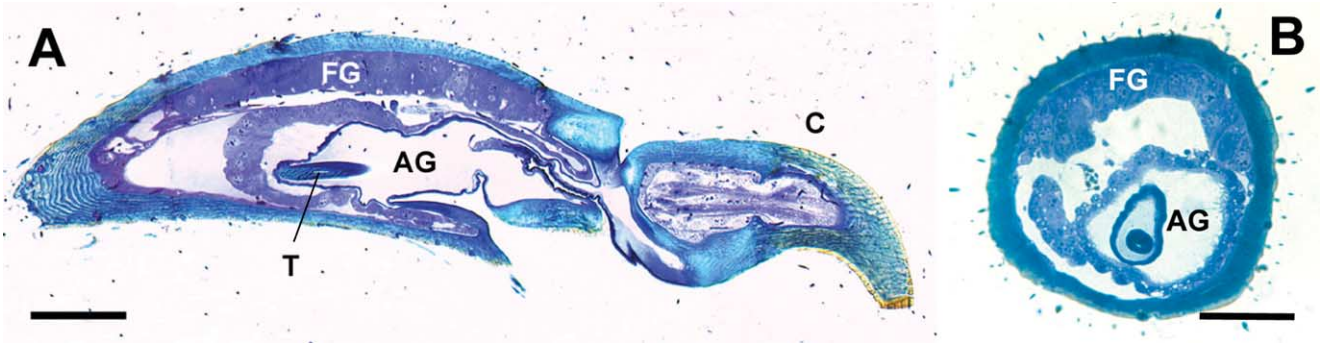

Fig. 5. Semithin parasagittal (A) and cross section (B) through hindleg pretarsus, showing conspicuous epithelium of dorsal footprint gland (FG). AG, arolium gland; C, claw; T, tendon of pretarsus. Scale bar 50 μm.

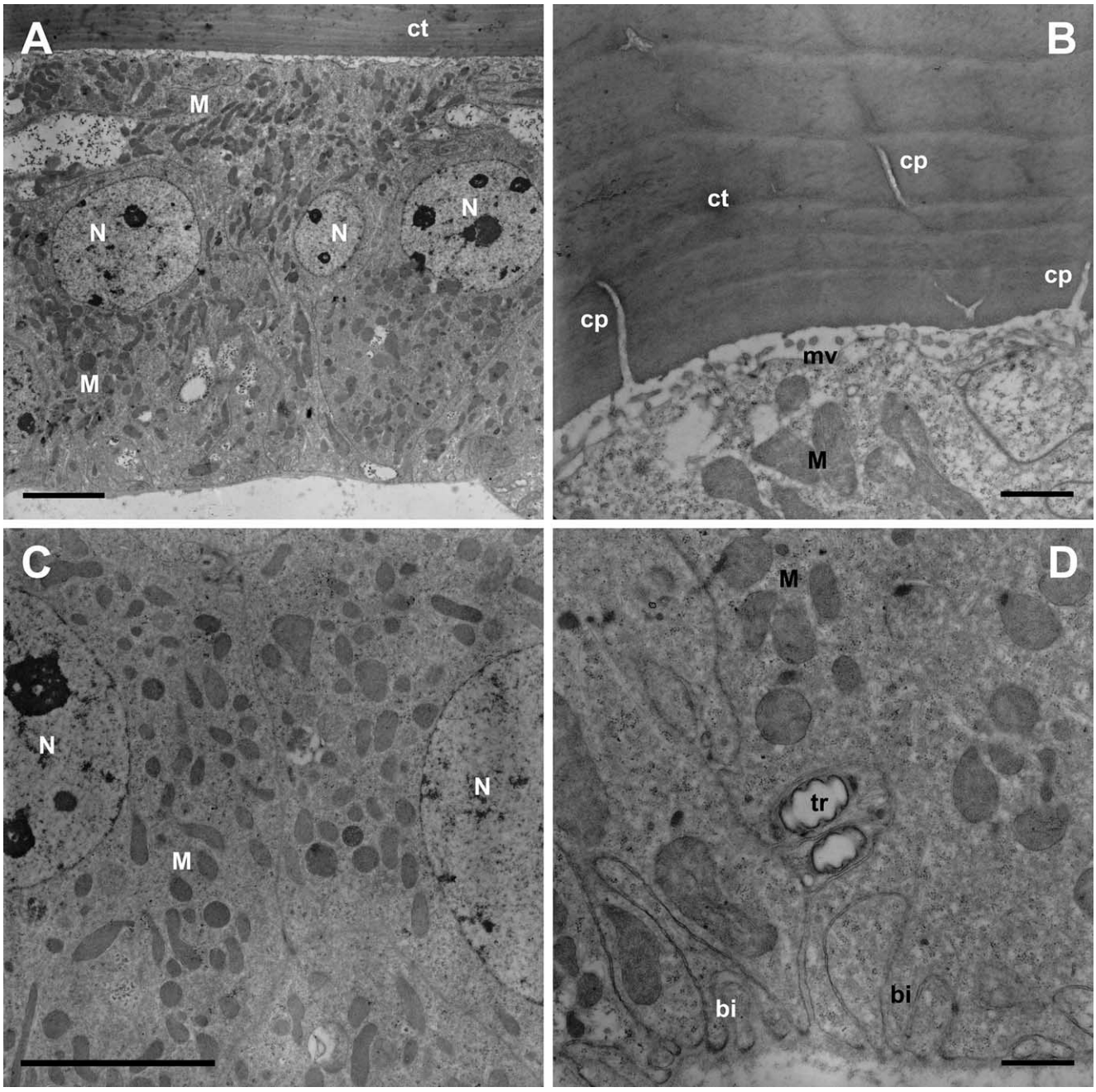

Fig. 6. Electron micrographs of footprint gland epithelium. A. Epithelium and inner portion of pretarsal cuticle; note the abundance of mitochondria (scale bar 5 μm), B. Detail of apical region showing irregular microvilli and cuticular pores (scale bar 1 μm), C. Part of cytoplasm with abundant mitochondria (scale bar 5 μm), D. Basal region of epithelium showing conspicuous invaginations of cell membrane and tracheoles penetrating between cells (scale bar 1 μm). bi, basal invaginations; cp, cuticular pores; ct, cuticle; M, mitochondria; mv, microvilli; N, nucleus; tr, tracheoles.

(1989a) mentioned 'a dense pattern of pore capillaries penetrating the cuticle of the ventral fifth tarsomere (…) that are absent in the dorsal part of the cuticle', but they did not provide any data on the presumed structural link between the dorsally occurring epithelium and the ventrally occurring pores. We could not find any such ventral pore channels in *A. reclinata*, but we did find them in the dorsal cuticle overlaying the footprint gland epithelium. The occurrence of narrow pore channels in the cuticle directly covering the glandular epithelium makes sense, as it carries the secretory products to the outside (Quennedey, 1998). Conspicuous regions with numerous minute pores have been found overlaying the metatibial gland in ants of the doryline section (Hölldobler et al., 1996; Billen, 1997) as well as the femoral and tibial glands of *Strumigenys* species (Billen et al., 2000). On the other hand, it may appear surprising that trail substances to be deposited onto the substrate are produced in a dorsally located gland. Careful observation,

however, revealed that during trail-laying each touch-down phase of the hindlegs was characterized by a twisted position of the pretarsi. This explains both how secretory products can be deposited onto the substrate and why the trail is formed by discontinuous marks rather than as a continuous line (the latter would have been the case if trail laying were done through leg dragging). The slightly more irregular pattern produced by trail laying workers can be understood by the combined prints of the regular walking pattern by the first and middle legs with those of the trail depositing hindlegs.

The structural organization of the footprint gland in *Amblyopone* ants corresponds to the class-1 epithelial gland following the standard classification of Noirot and Quennedey (1974). In this class, the secretory cells are nothing more but modified epidermal cells with a pronounced glandular activity. The occurrence of numerous infoldings of the basal cell membrane as well as apical microvilli illustrate the transport of secretory material, which is then discharged through the cuticular pores. It is surprising that no pore openings could be seen on the dorsal pretarsal surface, which may indicate that secretion crosses the uppermost region of the cuticle via diffusion. The cytoplasmic composition with abundant mitochondria and a clear tracheolar supply are indicative for a high metabolic activity. The apparent absence of granular endoplasmic reticulum makes it unlikely that proteinaceous substances are elaborated, although the chemical nature of the secretory products remains unknown.

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