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SECRETORY CYCLE OF THE DUFOUR'S GLAND IN WORKERS OF THE BUMBLE BEE *BOMBUS TERRESTRIS* L. (HYMENOPTERA: APIDAE, BOMBINI)

by

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ABSTRACT

The Dufour's gland of *Bombus terrestris* workers, of different ages and with varying degrees of ovary development, was studied with the aim to verify its involvement in reproduction. Measurements of the diameter and the length of the gland were made using an ocular micrometer adapted to a microscope. Transmission electron microscopy was used to study the morphology of the secretory cells and to analyze the secretory cycle. The glandular cells were considered to be near type II cells of NOIROT and QUENNEDEY (1974), a type that has not been described before in Hymenopterans. The results show that there is a correlation between the degree of ovary development and Dufour's gland activity of workers. The diameter of the gland and the secretory cell activity increased with increasing oocyte size in the ovary. Regressive conditions of the gland were observed, which are probably related to increasing worker age. To elucidate the production and releasing process of the secretion and to establish its precise function, a comparative analysis of the secretion process of the Dufour's gland of queens and workers is needed.

KEY WORDS: *Bombus terrestris*, Bumble bee, Dufour's gland, egg marking, workers.

INTRODUCTION

The Dufour's gland (DUFOUR, 1835) is found in close association to the sting apparatus of all female hymenopterans (BORDAS, 1894; LELLO, 1971). However, ultrastructural studies have revealed that the gland in bees opens into the dorsal vaginal wall (BILLEN, 1987), suggesting a relationship with reproduction.

The glandular secretion contains several long-chain hydrocarbons (HERMANN & BLUM, 1981) as well as volatile substances, such as esters and macrocyclic lactones (HEFETZ *et al.*, 1979). In many bee species, the composition of the secretion may show inter- and intra-specific variations (HEFETZ, 1987; HEFETZ *et al.*, 1993). In those social bees with caste

differentiation, as in *Apis*, the compounds of the Dufour's gland secretion can differ between the castes, forming a caste-specific pheromone set (KATZAV-GOZANSKY *et al.*, 1997). The Dufour's gland in *Apis* is larger in the queens than in the workers (CRUZ-LANDIM, 1967). Furthermore, the secretion in the queen bees contains wax-type esters that only appear in those workers that, upon orphanage, differentiate into egg-laying workers (KATZAV-GOZANSKY *et al.*, 1997). Hence, the caste-specific differences in the secretion of the Dufour's gland of the honey bee have induced some authors to suppose that this gland is involved in the production of egg-marking pheromones, whose function it is to protect the eggs of the queen during worker inspections (RATNIEKS, 1993, 1995; KATZAV-GOZANSKY *et al.*, 1997).

Nevertheless, the Dufour's gland serves many functions. In some solitary bees, the gland is the source of a hydrophobic material used as brood cell lining (CANE, 1981; KRONENBERG & HEFETZ, 1984, HEFETZ, 1987) and, in other species, it can mediate sexual behaviour (SMITH *et al.*, 1985) and nestmate discrimination (HEFETZ, 1990). Besides this, the gland might produce nest entrance markers (SHIMRON *et al.*, 1985), trail pheromones (VINSON *et al.*, 1978), or an adhesive substance placed onto the egg before it is deposited at the bottom of the brood cell (BILLEN, 1987).

The bumble bee colony cycle is characterized by three stages: (A) the solitary queen produces a small number of eggs from which workers emerge. This leads to (B), the social phase. In this phase workers are able to generate eggs, but they do not lay them until the queen loses her dominance (DUCHATEAU & VELTHUIS, 1989). The loss of dominance (C) results in competition for egg-laying opportunities, including aggression among workers and between workers and the queen (DUCHATEAU & VELTHUIS, 1988). This latter stage may be caused by changes in the pheromonal output of the queen (VAN HONK & HOGEWEG, 1981). Although much progress has been made in the elucidation of the various roles of the Dufour's gland in several species of social bees, its real function is still unknown. The purpose of this study was to investigate a possible correlation between the degree of development of the Dufour's gland and the degree of activation of the ovary of *Bombus terrestris* workers, and thus establish a functional link with reproduction.

MATERIAL AND METHODS

Measurements of the length and the diameter of the Dufour's glands of workers

The length and the diameter of the Dufour's gland of newly emerged and egg-laying *Bombus terrestris* L. workers were measured, as were that of workers in various intermediate stages of ovary development.

The Dufour's gland of workers was measured before the developmental stages of the ovaries (0-IV) of the individuals were checked and classified according to DUCHATEAU & VELTHUIS (1989). Newly emerged and egg-laying workers were collected from the colony and dissected directly in fixative solution. Likewise, intermediate stages of ovary development were sampled at random and the glands were measured using an ocular micrometer adapted to a microscope.

To verify the differences between the worker groups, variance analysis (Scheffé F-test) was used with a 5% level of significance.

Transmission electron microscopy (TEM)

The glands were fixed in Karnovski fixative for TEM (2.0% glutaraldehyde and 4% paraformaldehyde in 0.2 M phosphate buffer at pH 7.3), washed twice in buffer, stained with 2.0% uranyl acetate in 10% alcohol, and post-fixed in 1% osmium tetroxide in the same buffer. Dehydration was performed in a series of increasing concentrations of acetone, and embedding in Epon resin, followed by the usual procedures. Thin sections were cut with a diamond knife, contrasted with lead citrate, and examined in a Philips transmission electron microscope. Some thick sections were stained with methylene blue-azur II for light microscopy.

RESULTS

The Dufour's gland of *B. terrestris* workers is a slender tube with a dilated middle-distal region, that ends blindly in the distal extremity and opens into the dorsal vaginal wall. The measurements of the diameters were made in the middle-distal region (Fig. 1A). The length of the Dufour's gland of the various groups of workers was statistically equal, about 3.0-3.6 mm, while the diameter varied significantly (Table I). Newly emerged workers and workers with ovaries in stages 0 and I (group I) had a Dufour's gland with the smallest diameter (about 0.2 mm). Workers with ovaries in stages II and III (group II) had an intermediate gland diameter (about 0.3 mm) while the glands of workers with ovaries in stage IV as

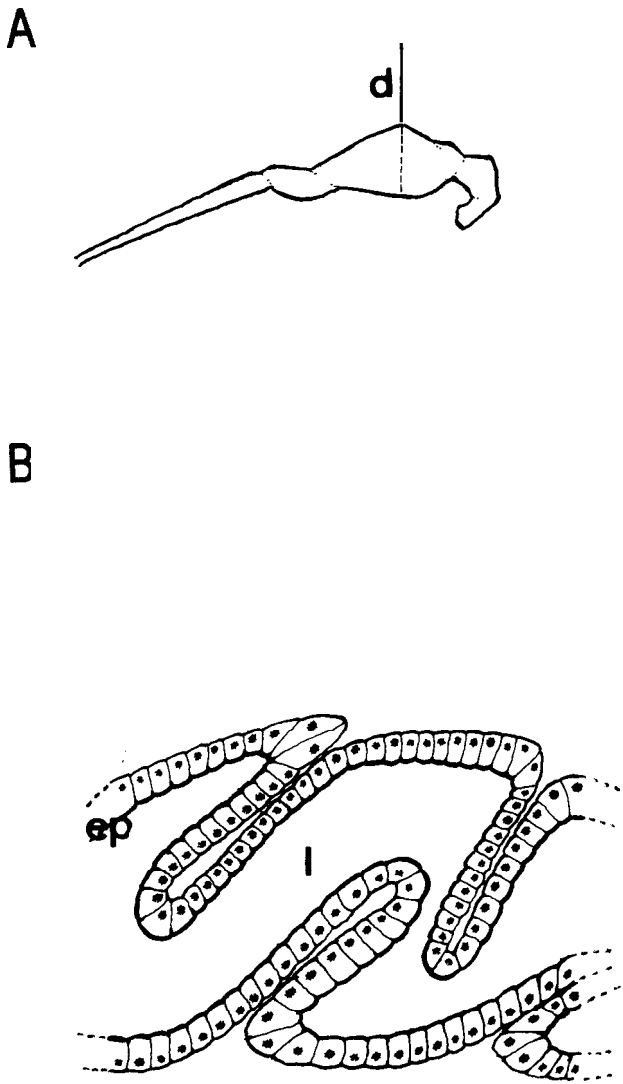


Fig. 1. Schematic representation of the Dufour's gland of a *Bombus terrestris* worker. A. The external aspect of the gland, resembling a slender tube with a dilated middle-distal portion (d). The measurements of the diameter were made in the dilated portion. B. Interspersed infolding pattern of the epithelial wall of the Dufour's gland of a worker, showing the monolayer epithelium (ep) and lumen (l), lined by a thin cuticle.

TABLE 1

Statistical values and variance analysis of the length and the diameter of the Dufour's gland of *Bombus terrestris* L. (Hymenoptera: Apidae, Bombini) workers of different ages and with different degrees of ovary development (after DUCHATEAU & VELTHUIS, 1989).

Workers	Number of individuals	Length (mm)			Diameter (mm)		
		mean	SD*	F-test**	mean	SD	F-test
Newly emerged	10	3.30	0.44	A	0.26	0.07	A
With ovary in stage 0	10	3.21	0.71	A	0.26	0.06	A
With ovary in stage I	12	3.03	0.95	A	0.26	0.10	A
With ovary in stage II	10	3.60	0.52	A	0.33	0.09	AB
With ovary in stage III	12	3.25	0.88	A	0.31	0.07	AB
With ovary in stage IV	22	3.60	0.83	A	0.40	0.11	B
Egg-laying worker	4	3.60	0.59	A	0.47	0.03	B

* Standard deviation, ** Sheffé test, variance analysis, with 5% level of significance.

well as egg-laying workers (group III) had the largest diameter (about 0.4 mm). The differences between these three groups of workers were significant (Table I).

Assuming that the gland diameter was the result of the quantity of secretion present in the glandular lumen, thus reflecting indirectly the physiological condition or the secretory activity pattern of the glandular cells, workers were divided into three groups according to the statistically significant differences of the diameter: group I — initial stage (IS), group II — intermediate stage (INS), and group III — highly active stage (HS). A regressive stage (RS) of the gland was also observed. These macromorphological differences were also reflected in distinctive ultrastructural features.

Histologically, the glandular tube had a single layer of epithelial cells; the epithelium was folded, forming an interspersed infolding pattern. The secretory cells were flat, cuboidal, or columnar (Fig. 1B), depending on the activity or developmental phase of the gland. A muscle cell sheath surrounded the gland, but did not form a closed envelope around the epithelium. The muscle fibers were of the insect visceral type, with a sarcomere length of about 0.5 μm (Fig. 2A).

The secretory cells of the Dufour's gland are of ectodermal origin, secreting a cuticular lining at the luminal surface (about 0.15 μm thick, Fig. 2B). Close examination of the cuticle revealed a thin electron-dense epicuticle (about 10 nm thick), a much thicker and more electron-lucid procuticle, in which it was often possible to discern a fibrillar arrangement, and an incipient exocuticle, an electron-dense band about 0.05 μm thick close to the epicuticle (Fig. 4C).

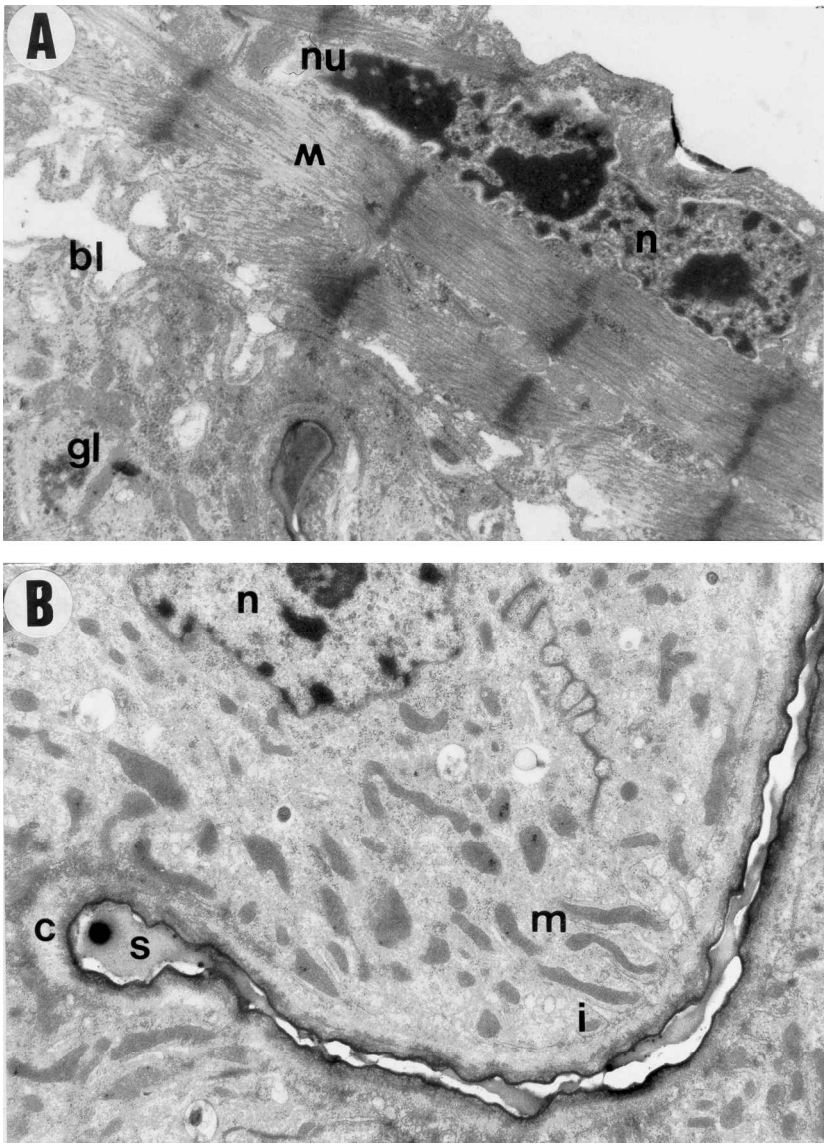


Fig. 2. Transmission electron micrographs (TEM) of the Dufour's gland cells of a worker in the IS. A. Basal side of the gland (gl) showing the thin, low electron-dense basal lamina (bl), the absence of plasmic membrane infoldings, and the outer muscle (M) (× 62,500). B. Apical face of the cell showing the cuticle (c) lining the collapsed lumen, where little secretion (s) is present. Note the few apical invaginations (i) of the plasmic membrane (× 31,500). m = mitochondria, n = nucleus.

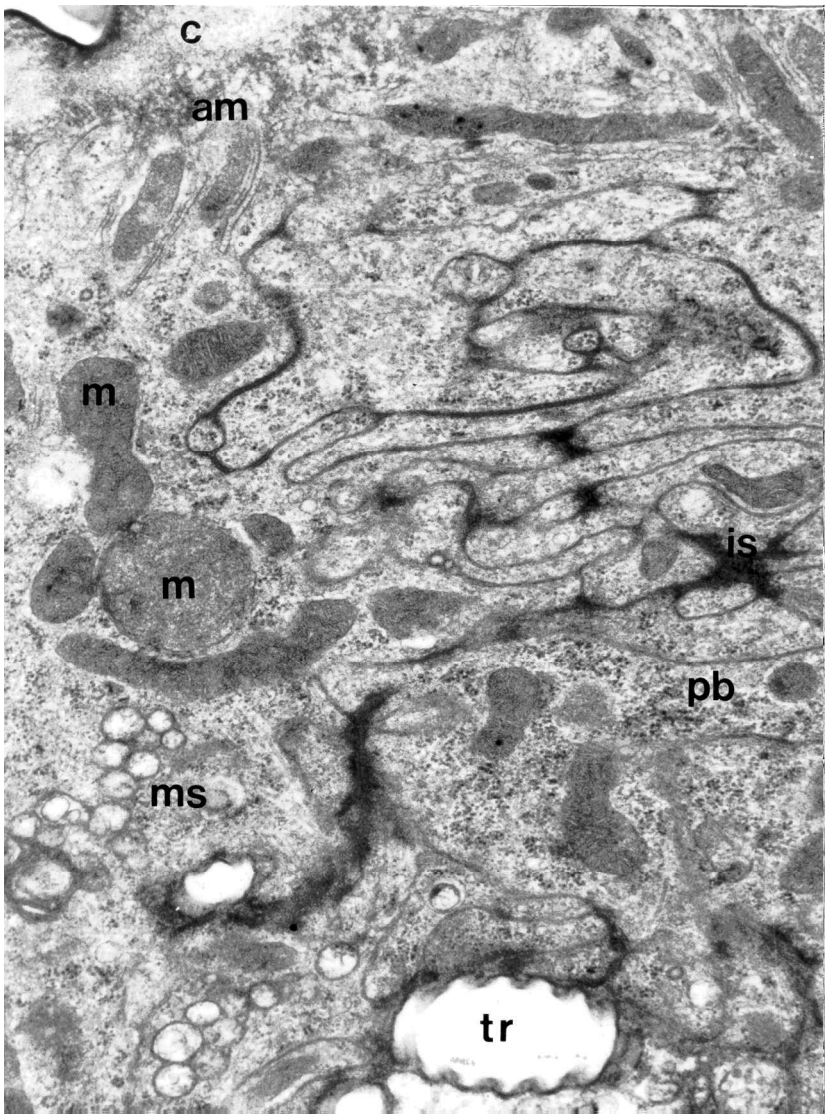


Fig. 3. Magnification of the apical surface showing some subcuticular accumulation of amorphous electron-dense material (am), polymorphic mitochondria (m), polyribosomes (pb), and membranous structures (ms). Note the electron-dense material in the intercellular spaces (is) (× 80,000). c = cuticle, tr = tracheole.

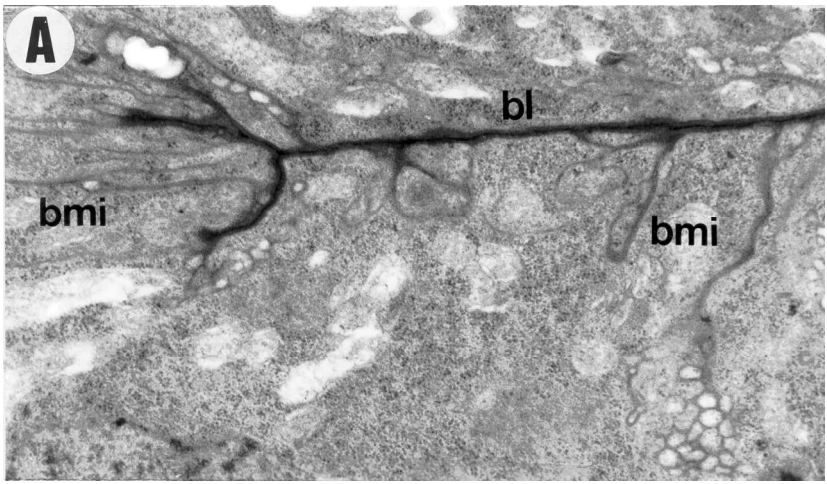


Fig. 4. TEM micrographs showing morphological signs of activity in the cells of a worker in the IS. A. Electron-dense basal lamina (bl) advancing between adjacent cells and basal plasmic membrane infoldings (bmi) filled with electron-dense material (× 62,500). B. Mitochondria (m) with very electron-dense matrix and polymorphic granules (pg) of unknown nature (× 90,000). C. Increase of the subcuticular space (sc), that nevertheless appears empty, and swelling of the cuticular infoldings (i) (× 125,000). c = cuticle, g = Golgi apparatus, l = lumen, mt = microtubules, pb = polyribosomes, va = vacuole.

The active glandular cells were usually columnar and had a basal nucleus, which was often oval with dispersed chromatin and one or two large nucleoli. Smooth endoplasmatic reticulum (SER) and mitochondria were among the most prominent organelles (Fig. 2B). The mitochondria changed in their morphology, their matrix electron density, and their distribution pattern within the cell during the activity cycle of the gland. In some cells, the mitochondrial matrix was very electron-lucid, which made them hard to observe. Inclusions in the mitochondrial matrix showed up more frequently in the secretory cells of those workers having ovaries in more advanced developmental stages (Fig. 6B). Such inclusions were rare in the early stages. The mitochondria often had a random distribution, but could aggregate apically or basally in the cells (Fig. 2B).

The plasmic apical membrane of the glandular cells was folded; the invaginations were usually packed very closely and associated with elongated mitochondria (Fig. 2B). Some specialized invaginations had a single spherical mitochondrion inside (Fig. 4C). Extensions of SER often occurred inside individual invaginations. On the other side of the cell, the basal lamina produced deep infoldings between the adjacent cells. The frequently electron-dense basal lamina sometimes produced electron-dense

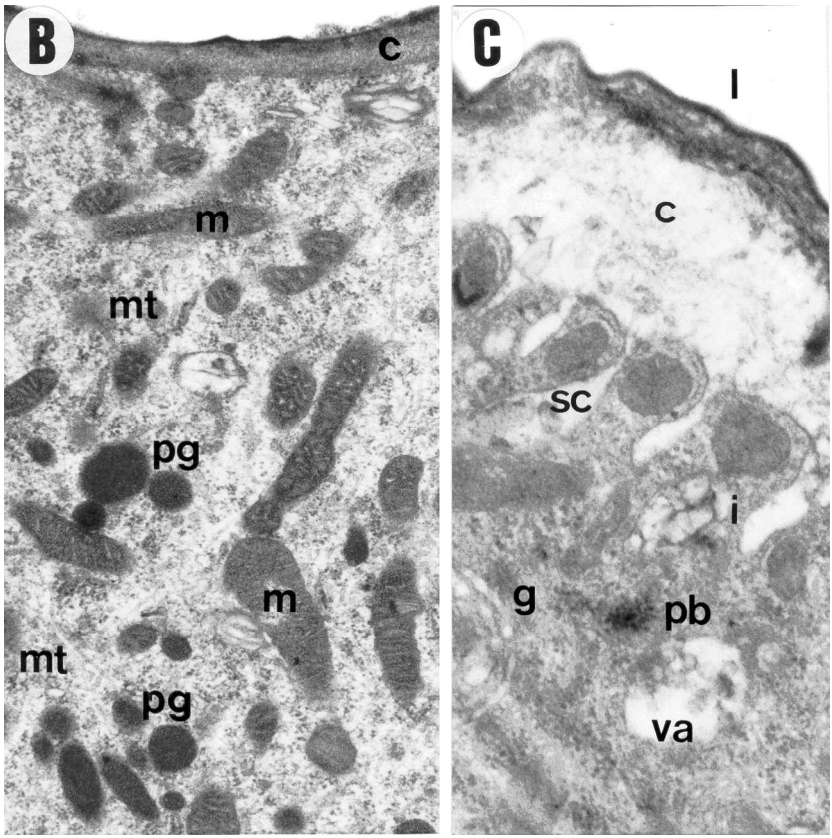


Fig. 4. (Continued).

ramifications penetrating deeply into the cell, accompanied by the plasmic membrane. Such basal plasmic membrane invaginations also increased in number and electron density in workers with developed ovaries (compare Figs. 4A and 7A).

The lumen of the gland was often collapsed in young workers and wide open in old ones or in those with developed ovaries. Secretion of a lipidic nature was always present and the amount was larger in workers with developed ovaries (compare Figs. 2B and 5C). Tracheoles were occasionally seen penetrating between adjacent gland cells (Fig. 3). It was quite common to find a very developed tracheole network in the glands of young individuals.

The three groups (IS, INS, and HS) followed a chronological order of increasing secretory activity related to ovary development and bumble bee

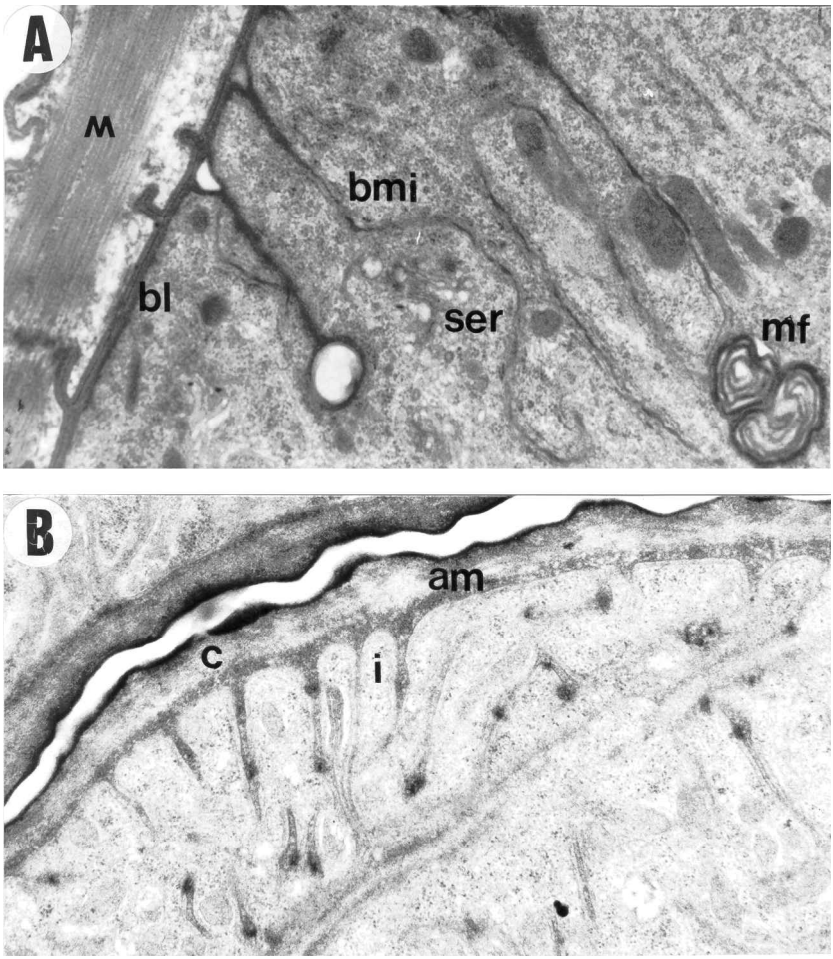
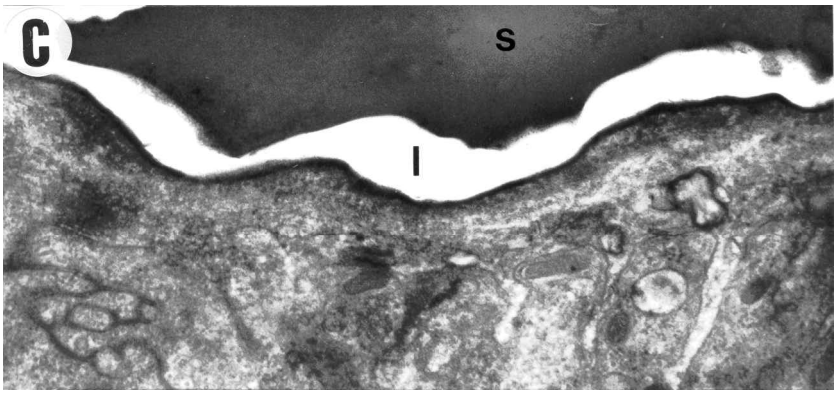


Fig. 5. TEM micrographs of the Dufour's gland of a worker in the INS. A. Very electron-dense basal lamina (bl) showing pegs toward the outer surface. The plasmic membrane invaginations (bmi) are filled with electron-dense material and lined by smooth endoplasmic reticulum (ser) (× 62,500). B. The apical invaginations (i) are filled with amorphous material (am) that also accumulates below the cuticle (c) (× 62,500). C. The lumen (l) is enlarged and contains secretion (s) (× 80,000). mf = myelin figures, M = muscle.

colony cycle. The RS was found during all phases of the colony cycle and was probably related to the age of the worker. In the IS, the Dufour's gland had the smallest diameter (Table I) and the secretory cells apparently produced only a small quantity of secretion. The secretory cells had few



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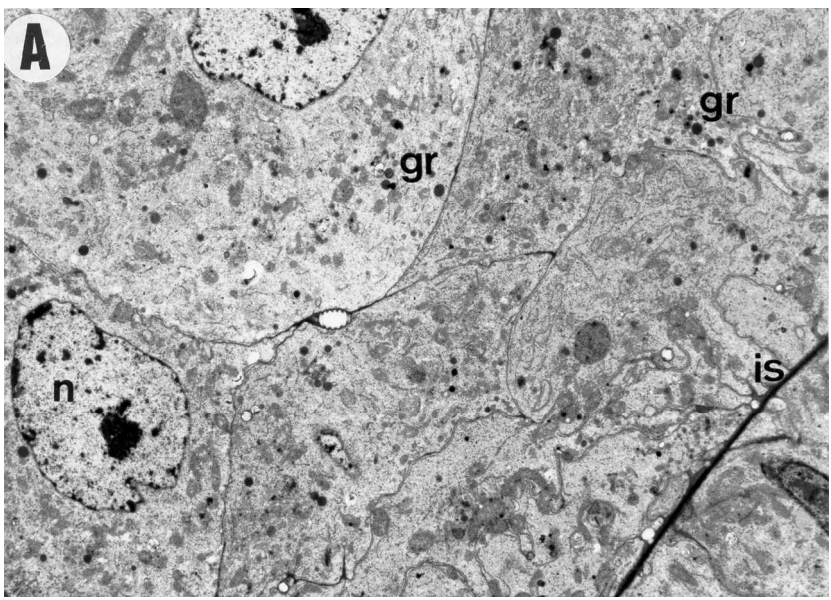

Fig. 6. TEM micrographs of the Dufour's gland of a worker in the HS. A. Note the large amount of small electron-dense granules (gr), that may represent a endogenous secretion (× 80,000). B. Note the nuclei (n) with very dispersed chromatin and large nucleoli (nu) with active features. Note also the presence of electron-dense deposits in the mitochondrial (m) matrix (× 50,000). C. The figure shows smooth endoplasmic reticulum (ser) and myelin figures (mf) (× 50,000). is = intercellular space.

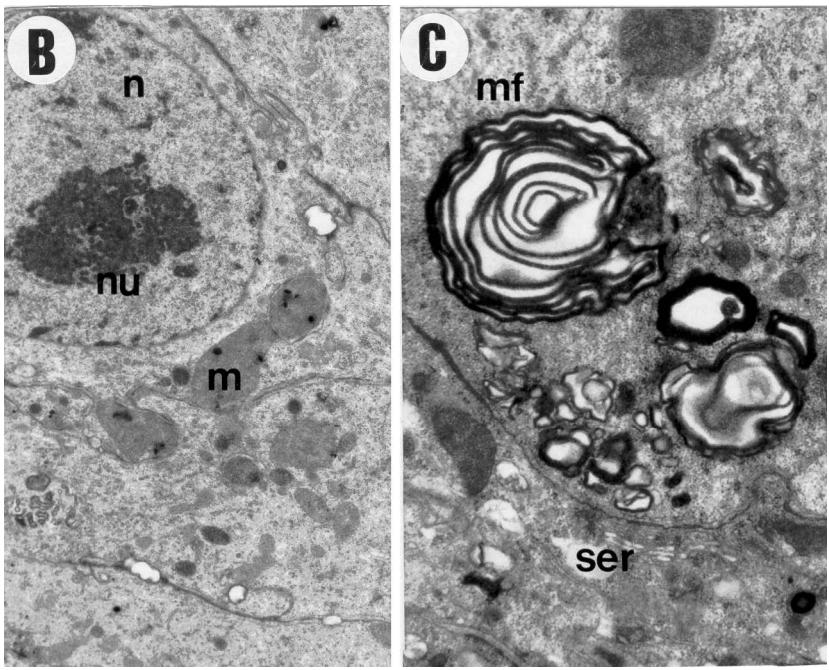

Fig. 6. (Continued).

apical invaginations (Fig. 4B). When these invaginations were present, the space between the cells and below the cuticle was often empty (Fig. 4C), indicating low release rates of secretion to the lumen in this stage. The cytoplasm had abundant mitochondria and a few inclusions (Fig. 4B) of undetermined nature.

The ovaries of the workers were in stage II or III in the INS. There was an increase in the number of basal and apical invaginations (Figs. 5A, B). The subcuticular space was enlarged and there was more secretion below the cuticle and in the lumen than in the IS (Figs. 5B, C). The procuticle was also more electron-dense in this stage. The release of secretion appeared to be intensified (compare Figs. 2B, 4C, 5B, C).

In the HS, the Dufour's gland had the largest diameter and was found in workers with ovaries in stage IV as well as egg-laying workers. In this stage, the cells appeared to have a high secreting activity. The cytoplasm had abundant, very electron-dense, small granules (Fig. 6A) and the nuclei with very dispersed chromatin had large nucleoli with active features (Figs. 6A, B). Electron-dense material in the intercellular space (Fig. 6A), and small electron-dense deposits in the mitochondrial matrix

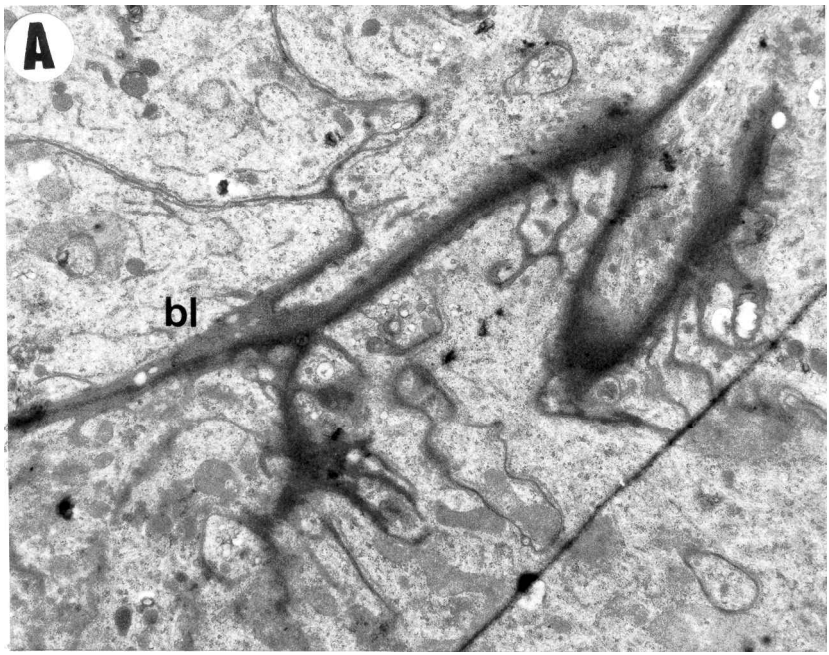

Fig. 7. TEM micrographs of the Dufour's gland of a worker in the HS showing features suggestive of the uptake of material from the haemolymph by the secretory cells. A. Note the very electron-dense basal lamina (bl) (× 31,500). B. Note the outer droplets (d) of material with an electron density similar to that of the basal lamina (bl) (× 31,500). gl = basal side of the gland, M = muscle, bmi = basal membrane invagination.

(Fig. 6B) were observed. Myelinic bodies appeared more frequently, perhaps because of residues left by secretion elimination (Fig. 6C). Apical and basal invaginations were present and ramifications of the basal lamina infoldings were more prominent (compare Figs. 4A and 7A). Large extracellular electron-dense droplets appeared just below the basal lamina infoldings (Fig. 7B).

The RS did not occur in a chronological sequence of the colony cycle. This stage probably depends on worker age, because it was found in an old worker with ovary in stage I, where the glands should present IN aspects (compare Figs. 2B and 8A, B). The secretory cells in the RS showed many reabsorptive features, few apical and basal invaginations, and very few organelles. The oval nuclei were large, with condensed chromatin (Figs. 8A,B).

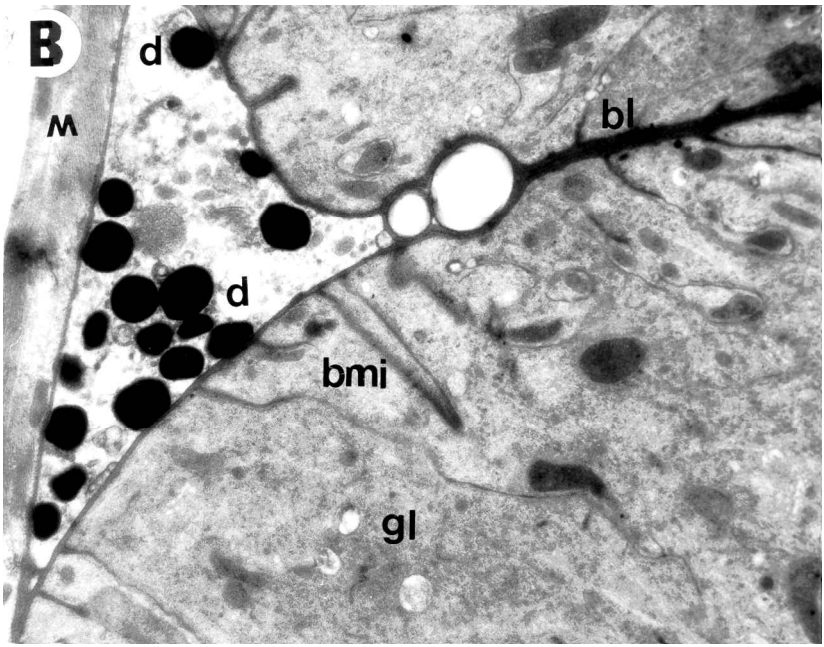

Fig. 7. (Continued).

DISCUSSION AND CONCLUSIONS

According to the classification of the glandular cells of insects by NOIROT & QUENNEDEY (1974), the cells of the Dufour's gland could be class I cells, where the secretion is directly eliminated through the cuticle. The increase in area of the cell subcuticular plasmic membrane, produced by the increase of apical invaginations, could be taken as evidence of increased secretion during the Dufour's gland cycle. This interpretation is also supported by the amount of material that is accumulated below the cuticle in the INS and the HS. However, consistent production of secretion by the gland cells was not observed. One explanation for this may be that the secretion produced is continuously eliminated and therefore does not accumulate in the cells.

Another possibility is that the secretion is of exogenous origin, and that the gland cells only transmit it (class II of NOIROT & QUENNEDEY (1974)). This means that the secretion is produced elsewhere, probably in the fat body or oenocytes. Nevertheless, secretory class II cells do not exactly represent the type of glandular cell found in the Dufour's gland, because we could not consistently observe cell types other than the

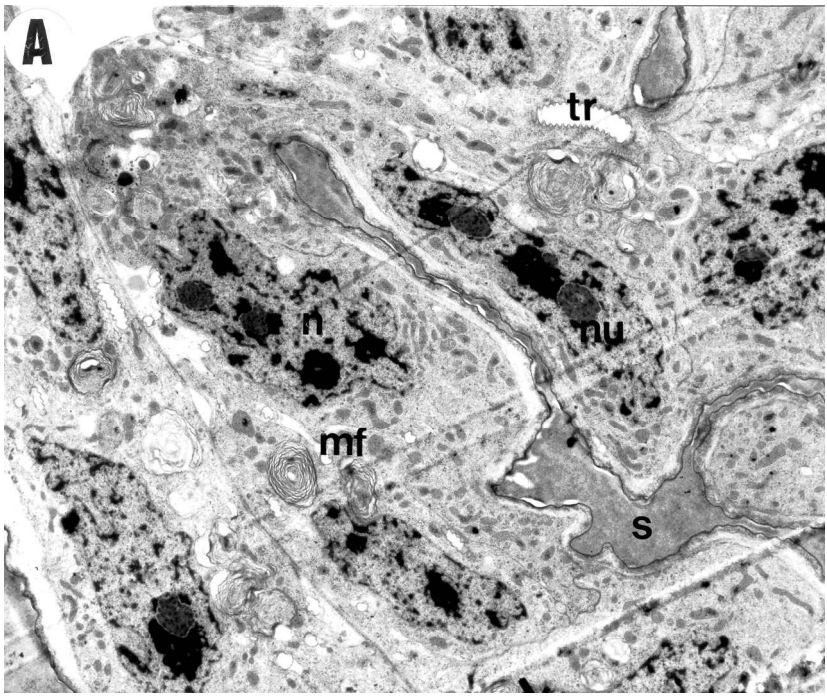

Fig. 8. TEM micrographs of the glands in the involutive stage. Note in A (× 15,750) and B (× 31,500), the nuclear (n) and nucleolar (nu) condensation, the lower cells, and the numerous myelin figures (mf). s = secretion, tr = tracheole.

Dufour's gland epithelium, to which we could assign the synthesis of the secretion. Instead, it seemed that the material absorbed by the epithelial cells was coming directly from the haemolymph. As HEFETZ *et al.* (1993) observed, the secretion composition may vary with the concentration of the food. Furthermore, oenocytes were not observed in association with the Dufour's gland in *B. terrestris*. In this line of reasoning, the Dufour's gland could be considered both an organ of capture of exogenous substances of lipidic nature present in the haemolymph, and an organ of minor secretory activity. Type II secretory cells are, in fact, very uncommon in hymenopteran exocrine glands and, to date, have been found only in the sternal glands of termites (NOIROT & QUENNEDEY, 1974).

Indirect proof that there is external uptake of material is the paucity of secretory organelles and secretion vesicles in the cells, suggesting no evident intracellular production of secretion in the gland. Besides, in the INS and HS stages, the basal lamina invaginations and intercellular spaces

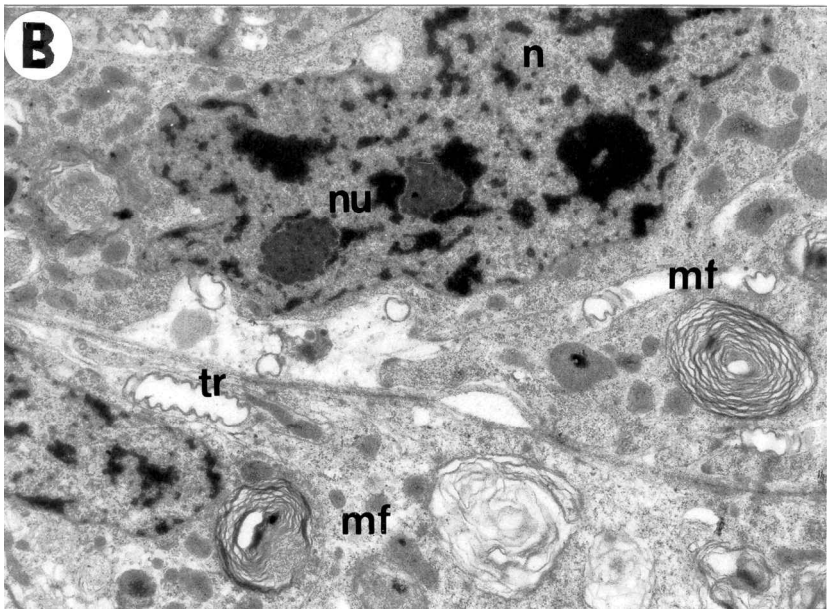

Fig. 8. (Continued).

were increased in number, deepness, ramification, and electron density. Furthermore, in every stage, but mostly in the HS, it was possible to see large extracellular droplets of electron-dense material below the basal lamina infoldings, similar to lipids, suggesting an uptake of this material by the glandular cells. The SER was poorly developed and the mitochondria were among the most numerous organelles found in the cytoplasm of the glandular cells. Perhaps this great quantity of mitochondria is involved in the energy supply for the uptake of the external secretion. The absence of granular endoplasmic reticulum reflects the non-proteic nature of the secretion of the Dufour's gland. The abundant polyribosomes and some Golgi apparati (GA) in the cytoplasm could indicate some modification of the secretion, directly by the GA and indirectly by the polyribosomes, the latter probably being involved in enzyme synthesis.

The increase in the diameter of the Dufour's gland reflects the accumulation of secretion in the lumen in certain stages. The fact that the diameter does not decrease in egg-laying workers might indicate a continuous production and use of the secretion as suggested before. The dilated distal-middle portion may function as a reservoir, although the glandular tube also has secretory aspects.

If the ovary developmental pattern of the three groups of workers is taken into consideration, it can be concluded that there is a relationship

between the Dufour's gland secretory cycle and ovary development. Workers with poorly developed ovaries (stages 0 and I) have a less active Dufour's gland, while workers with developed ovaries (stages III and IV) or those collected while laying eggs have a more active Dufour's gland with a larger diameter. The gland appears to produce only a small amount of secretion in the IS and does not release it, while in the INS and the HS, there might be more production and release of secretion, being most prominent in the HS.

The correlation of activity levels suggests that the Dufour's gland has some strategic function in reproduction. Comprehension of the secretion activity of the bumble bee queen is necessary to understand this function. In addition, histochemical and chemical analysis of the produced substances should be studied.

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