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# Ultrastructural study on host-guest relationships between anuran serous cutaneous glands and nematodes

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**Abstract** — We described guest nematodes in serous and lipid skin glands in two anuran species from the Old and New Worlds: *Rana camerani* and *Phyllomedusa hypochondrialis*, respectively. These glands are involved in three types of response: a) invaginations from the periluminal plasma membrane which accommodate the "round worms"; b) secretory release into such hosting chambers; c) migration of macrophages from the periglandular stroma to the secretory unit. There are no obvious signs suggesting the glands are harmed by the nematodes, nor are the latter affected by defence reactions, either secretory or cell-mediated, of their hosts. Nematodes are natural tracers which allow analysis of the inner space system (lumina as well as interstitia) in the glands. Furthermore, they provide the opportunity for functional characterisation of anuran serous glands under intrusion stress, revealing peculiar reactive traits, namely a pliable periluminal plasma membrane and inducible processes of secretory release.

**Key words:** Nematoda, *Phyllomedusa hypochondrialis*, *Rana camerani*, serous glands, skin, ultrastructure

## INTRODUCTION

Cutaneous serous glands in amphibians represent a diffuse exocrine system pertaining to the adaptive organ set that accompanied the transition of vertebrates from an aquatic to terrestrial habitat (Toledo and Jared 1995). These glands are interface organs between body and external environment, and consist of an intra-epidermal duct, a sub-epidermal intercalary tract (or neck, the stem compartment) and an intra-dermal secretory unit, ensheathed by a contractile layer of smooth myocytes or myoepithelial cells (Delfino 1991). Anuran serous units have a peculiar syncytial structure that develops during secretory cytodifferentiation processes in pre-metamorphic tadpoles (Delfino *et al.* 1988). Serous syncytia display a centripetal polarisation with the nuclei arranged at the periphery while the secretory deposits occupy the inner region. Secretory product storage is intracytoplasmic, as anuran serous glands posses an exiguous lumen at the level of, or just below, the intercalary tract.

Under the light microscope (LM), discrete secretory cells (adenocytes), have sometimes been detected between the neck and secretory syncytium (Delfino *et al.* 1990; 1992). Transmission electron microscope (TEM) studies revealed that they derive from stem cells (adenoblasts) of the intercalary tract, that differentiate during gland development (Delfino *et al.* 1988, 1994) or post-discharge gland rehabilitation (Delfino 1980) and will merge into the common cytoplasmic bulk of the syncytial secretory unit. Further individual cells have been described under the TEM in resting glands, inserted within the interstitium between the secretory and contractile compartments (Delfino 1980; Delfino *et al.* 1990; 1992; 1998; 1999a; Nosi *et al.* 2002). Pioneering LM studies (Faraggiana 1938a; 1939), complemented by ultrastructural findings (Delfino 1980; Delfino *et al.* 1992), indicate these cells to be macrophages

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migrating from the stromal environment, to remove metabolic waste and degenerated component parts of the secretory unit.

Although the above structural and ultrastructural findings confirm in full the fundamental syncytial arrangement of anuran serous units, we have recently detected under LM discrete structures within the common cytoplasmic bulk of serous glands. They were encircled by a light halo, and varied in shape from circular to elongated, with diameters about 10 μm. Since we consistently found these unusual features in species from different families (Hylidae and Ranidae) from South America and Asian Turkey respectively, we analysed them under the TEM to: a) ascertain whether they were cells (or pluricellular structures) pertaining to the secretory unit (thus opposing the concept of a serous syncytium) or other gland regions and, if they were not component parts of the organ, b) identify their nature and any relationships they may have with the gland. In either case, we expected that such unusual findings could improve our knowledge on the morpho-functional traits of these interface, secretory organs.

## MATERIAL AND METHODS

Adult specimens of *Rana camerani* Boulenger, 1886 were collected from the outskirts of Kars (Eastern Turkey) and specimens of *Phyllomedusa hypochondrialis azurea* Cope, 1862 near Resistencia (Chaco, Argentina). Preliminary fixation steps were performed respectively at the Department of Biology (Uludag University, Science and Art Faculty, Bursa, Turkey) and Departamento de Biología (Universidad Nacional del Nordeste, Corrientes, Argentina). In both cases skin strips (4 $mm^2$ in surface area) were removed from animals sacrificed with 0.2% chlorobutanol and treated (2 hr, 4°C) with an aldehyde mixture according to Karnovsky (1965). The tissue fragments were then washed with 0.1 M, pH 7 sodium cacodylate buffer (the same as the pre-fixative solution) and despatched in 2-4 ml of this buffer (with the addition of a drop of 3% glutaraldehyde) to the Dipartimento di Biologia Animale e Genetica (Università di Firenze, Italy). Here, the skin specimens were rinsed once more, reduced in size and post-fixed in 1% $OsO_4$ (90 min, 4°C), again using the sodium cacodylate buffer. After rinsing in this solution, the samples were dehydrated in graded ethanol, soaked in propylene oxide and infiltrated in Epon 812 to obtain flat blocks. The Epon blocks were cut with a NOVA LKB ultramicrotome into semithin (1-2 μm) and ultrathin (yellow-white, interference colour) sections. In addition, tissue samples were processed according to routine light microscopy schedule, using polystyrene as an embedding medium (Frangioni and Borgioli 1979). Along with 7μm thick, polystyrene sections (stained with the Mallory trichrome method), semithin sections (stained with buffered tolouidine blue) were used for preliminary light microscope examinations. Ultrathin sections were collected on 300 mesh, uncoated copper grids, then electron-dense stained in sequence with hydroalcoholic uranyl acetate and alkaline lead citrate solutions (saturated and 2 mg/ml, respectively). Finally, these samples were examined (80 kV) under a Siemens 101 electron microscope.

## RESULTS

Under the LM, most serous glands in *Rana camerani* specimens contain elongated, bowed structures (Figs. 1A and 1B), while a few of them store minute secretory granules (Fig. 1A). The stromal connective tissue (spongy dermis) is rich in cells, which tend to crowd around some serous glands (Fig. 1B). When observed under the TEM, serous gland content consists of dense granules with a spongy appearance, along with larger, complex structures, elliptical to circular in section, which exhibit a multicellular organisation (Fig. 1C). Component cells are arranged according to definite layers, and the innermost hold vesicular structures with a weak electron-dense content (Figs. 1C and 1D): the ultrastructural traits described below suggest that these elongated structures are nematodes, "round worms", hosted in serous glands. The serous gland secretory granules exhibit only slender halos around them, but nematodes are surrounded by dilated, translucent spaces, which continue into the syncytial cytoplasm with labyrinthine paths (Fig. 1D). In some instances, these large spaces contain thin sheaths (Fig. 1C) with a saw-edged profile in section shed by the body surface of the nematodes, which are provided with external, annulate reinforcements (Fig. 1D). These laminar structures are *exuviae* of the nematode hypodermis, provided with ring-shaped, cuticular micro-sculptures (see also Fig. 3D). However, smooth-surfaced areas of cuticle can also be detected (Figs. 1E, 2A and 2E), according to the moulting phase. In tangential section, nematodes can be seen to posses a peripheral

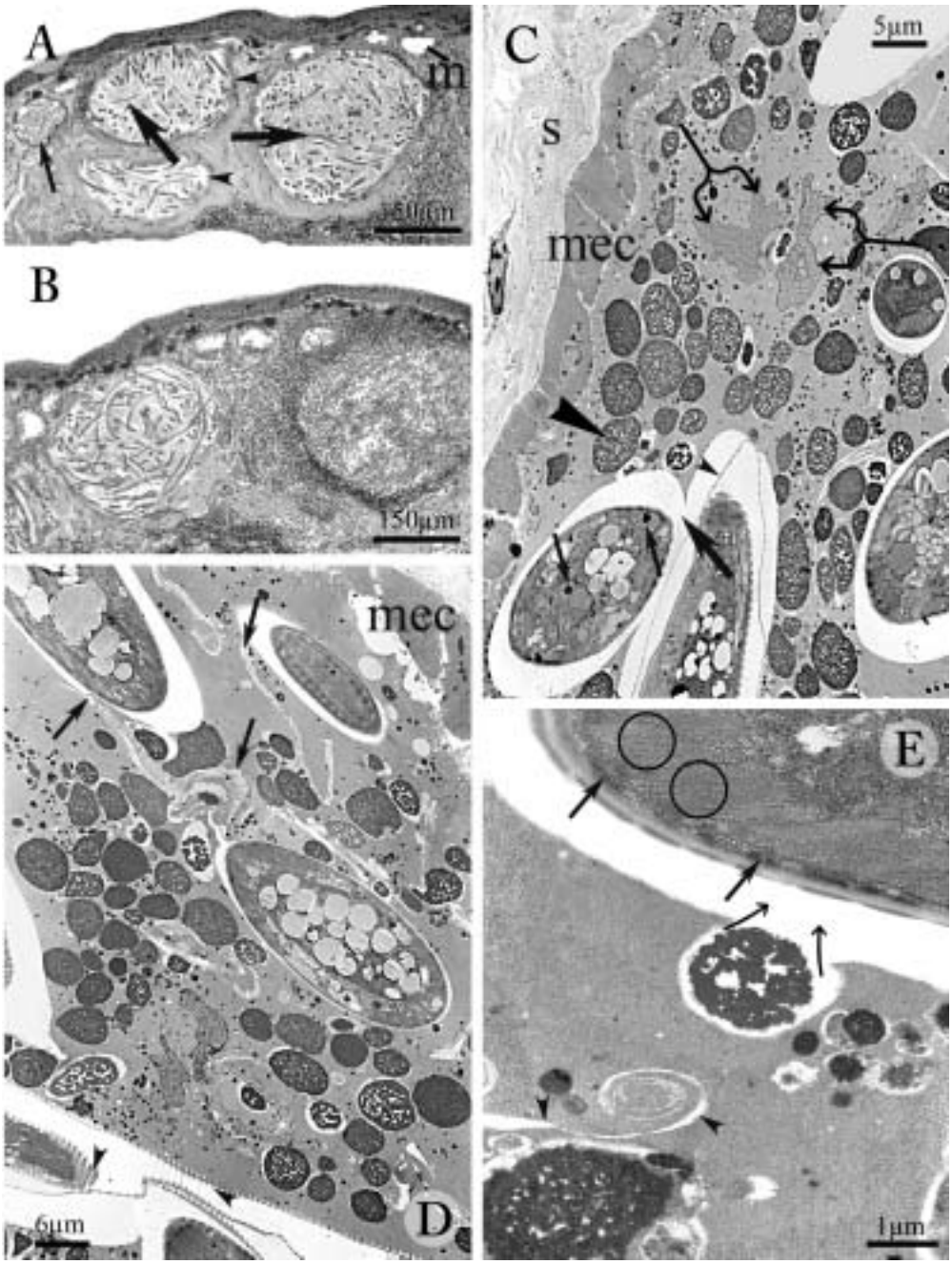


Fig. 1 — Cutaneous glands of *Rana camerani* observed under LM (A, B) and TEM (C-E). A: Notice serous glands containing elongated organisms (nematodes, large arrows), small mucous glands with empty lumina (m) and a serous unit holding typical, secretory granules (arrow). Superposition of two glands (arrowheads) is a section effect, due to their large sizes and irregular shapes. B: Contiguous serous glands: notice nematodes in the left one; the pattern at the right side, resembling a swarm of bees around their honeycomb, is due to crowding of macrophages. C: Peripheral portion of a serous gland. Notice the thick and continuous myoepithelium (mec), the gland stroma (s), spongy-like secretory granules (large arrowhead), irregularly shaped nuclei in the syncytium (forked arrows) and nematodes. These guests are encircled by wide halos, sometimes merging together (large arrow); small arrowhead points to a laminar structure apparently shed from a nematode, arrows to nuclei of component cells of a contiguous worm. D: The laminar structures resemble annulate sheaths of the guests (arrowheads). Arrows point to complex cavity patterns continuous with the spaces holding nematodes (compare with 2C). E: These compartments are involved in exocytotic, secretory release (bowed arrows). Straight arrows point to dense plaques anchoring contractile filaments of peripheral muscle cells to the body wall of the guest. Arrowheads indicate whorl-like evagination of the gland lumen (compare with 2B); circles include contractile filaments.

layer of muscle fibres, with filaments anchored to the thin hypodermis by dense plaques (Figs. 1E and 2E, compare with Fig. 3F).

The slender halos encircling individual secretory granules are often continuous with the larger spaces holding the guests, resembling a merocrine process (Fig. 1E). Higher magnifications show these secretory granules during release, when they flake off and give rise to fuzzy material (Fig. 2A). Around nematodes, characteristic whorls are recognisable, resulting from concentric alternation of cytoplasm layers with thin and transparent spaces (Figs. 1E and 2B). These alternations of cytoplasm layers and spaces also result in stalked profiles, where spaces are continuous with a complex cavity system holding both granules (Fig. 2C) and guests (Fig. 1D). Contiguous nematodes are separated only by thin cytoplasm partitions (Fig. 2D) or held together in the same compartment (Fig. 1C). Since nematodes are scattered throughout the syncytial cytoplasm of the secretory unit, they also occur in the peripheral regions, where the myoepithelial sheath wraps round the secretory compartment (Figs. 1C and 1D). Although nematodes are often found in close contiguity to the myoepithelium, they are always separated from the contractile cells by a thin cytoplasmic layer pertaining to the secretory syncytium (Fig. 2E). In these peripheral areas, the myoepithelium-secretory unit interstitium is easily distinguished from the halo round the guests: the former holds a thick network of slender processes, emanating from the facing cytoplasms, whilst the latter is empty and extremely regular in profile, due to parallel surfaces of cuticle and syncytium plasma membrane (Fig. 2E).

Data collected from *Phyllomedusa hypochondrialis* confirm previous findings on *Rana camerani* and provide new evidence on the ultrastrucural features of the guests as well as serous gland response. In this South American tree-frog, the glands hosting nematodes are located in the ventral skin and belong to the lipid producing type. Serial sections collected from U-shaped portions of the host body, show sequential profiles: first elliptical (Figs. 3A and 3B), then biconcave (Fig. 3C), later hour-glass in shape (Fig. 3D), and finally circular (Fig. 4D). These sections also reveal the main anatomical traits of the hosts: cuticle (fig 3 D), hypodermis (Fig. 3E), a single layer of longitudinal muscle fibres (Figs. 3A and 3F),?and internal organs (Figs. 3B and 3C). Higher magnifications confirm the ring-shaped microsculpture of the cuticle (Fig. 3D, compare with Fig. 1D) and show that the peripheral muscle cells include two distinctive cytoplasm zones (inner and outer) containing organelles and the contractile apparatus, respectively (Fig. 3F). This apparatus exhibits a regular pattern of thick and thin filaments; the latter converge toward the body wall, forming dense bands (Fig. 3F) also occurring in nematodes detected in *R. camerani* glands (Figs. 1E and 2E), Transverse sections of the cephalic end of guests show two parallel cuticular channels running along their longer axis, and pertaining to the oesophageal *procorpus* and the duct of a pharyngeal gland, respectively (Fig. 4A). Elaborate junctional complexes occur between the cells of the *procorpus* wall (Fig. 4A) and also involve cytoplasm processes with axonemal-like structures (Fig. 4B), possibly dendritic portions of receptor cells. Near its junction with the *procorpus*, the terminal tract of the gland duct exhibits a peculiar "end apparatus", consisting of complex cuticular structures, namely an internal pipe with a thick wall (Figs. 4B and 4C), and external radial reinforcements (Fig. 4C). Typical features, detectable at sub-cephalic levels, are paired aggregates of axial structures containing aligned microtubules, and resembling longitudinal nerve bundles (Fig. 4D).

As already observed in *Rana camerani*, glands hosting nematodes react towards these intruders by releasing their products; lipids are discharged as single droplets (Fig. 3E), or large aggregates (Figs. 3A, 3B, 3C and 5A). Despite their different amounts, secretory products are always released following the typical merocrine process.

At the periphery of the secretory unit, a large interstitium separates the syncytial cytoplasm from the discontinuous myoepithelium. As observed in *R. camerani*, slender cytoplasmic processes emanate from both gland component parts forming a thick, three-dimensional mesh (Figs. 3A, 3B, 5A, 5B and 5C). This interstice often holds migrating cells provided with a large (Fig. 5A), sometimes bilobate (Fig. 5B) nucleus. The distinctive shape of these nuclei is a vestige of migration across the myoepithelial cells, as suggested by the hour-glass shape the macrophages assume as they push their way into the secretory unit (Fig. 5C), seeking out the round worms (Fig. 5D).

## DISCUSSION

The occurrence of nematodes within serous or serous derived skin glands (such as the lipid producing type, DELFINO *et al.* 1998) provides an opportunity to analyse these secretory organs under peculiar "reactive" conditions. Although we have

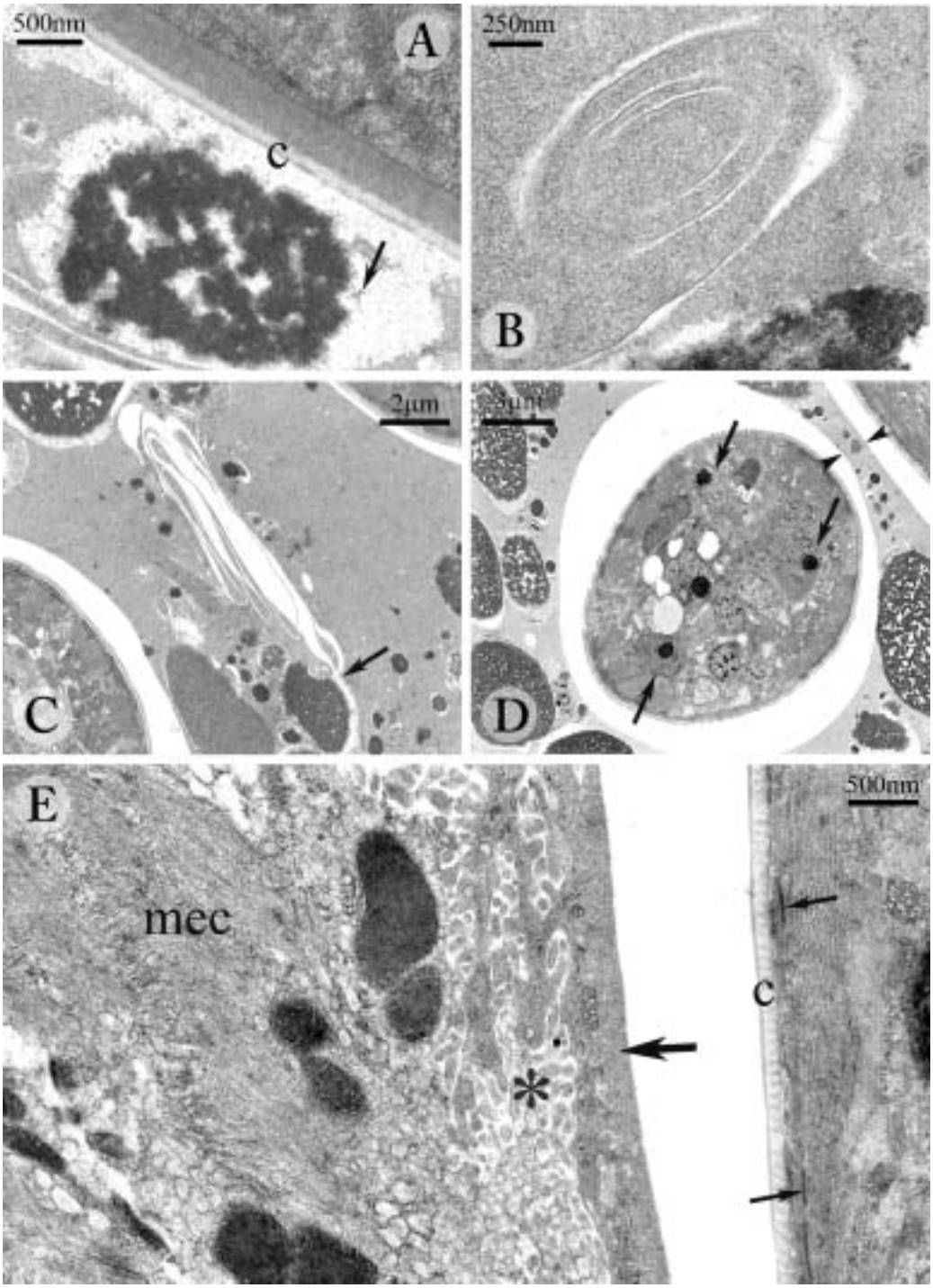


Fig. 2 — Details of serous gland-guest relationships in *Rana camerani*. A: Serous granule just released in the compartment holding a nematode: notice the fuzzy appearance of the granule cortex (arrow), similar to the thin material inside the cavity. As in 1E and 2E, this guest organism exhibits a smooth-surfaced cuticle (c). B: Detail of the whorl-like evagination in 1E (in a sequential section). C: This stacked pattern derives by alternation of thin cytoplasm layers and hollow spaces, continuous with a branch of the lumen holding a secretory granule (arrow, compare with Fig. 1D). D: Notice a thin cytoplasm screen (arrowheads) separating contiguous worms; arrows point to roundish nuclei of internal cells. E: Detail of gland periphery; from right to left one can notice: the nematode with peripheral, dense plaques (arrows) and smooth-surfaced cuticle (c, compare with 1E and 2A); the light compartment containing the guest; a layer of secretory syncytium (large arrow); the secretory-contractile interstice, characterised by a somewhat net-like pattern of thin cytoplasm processes (asterisk); and the myoepithelium (mec).

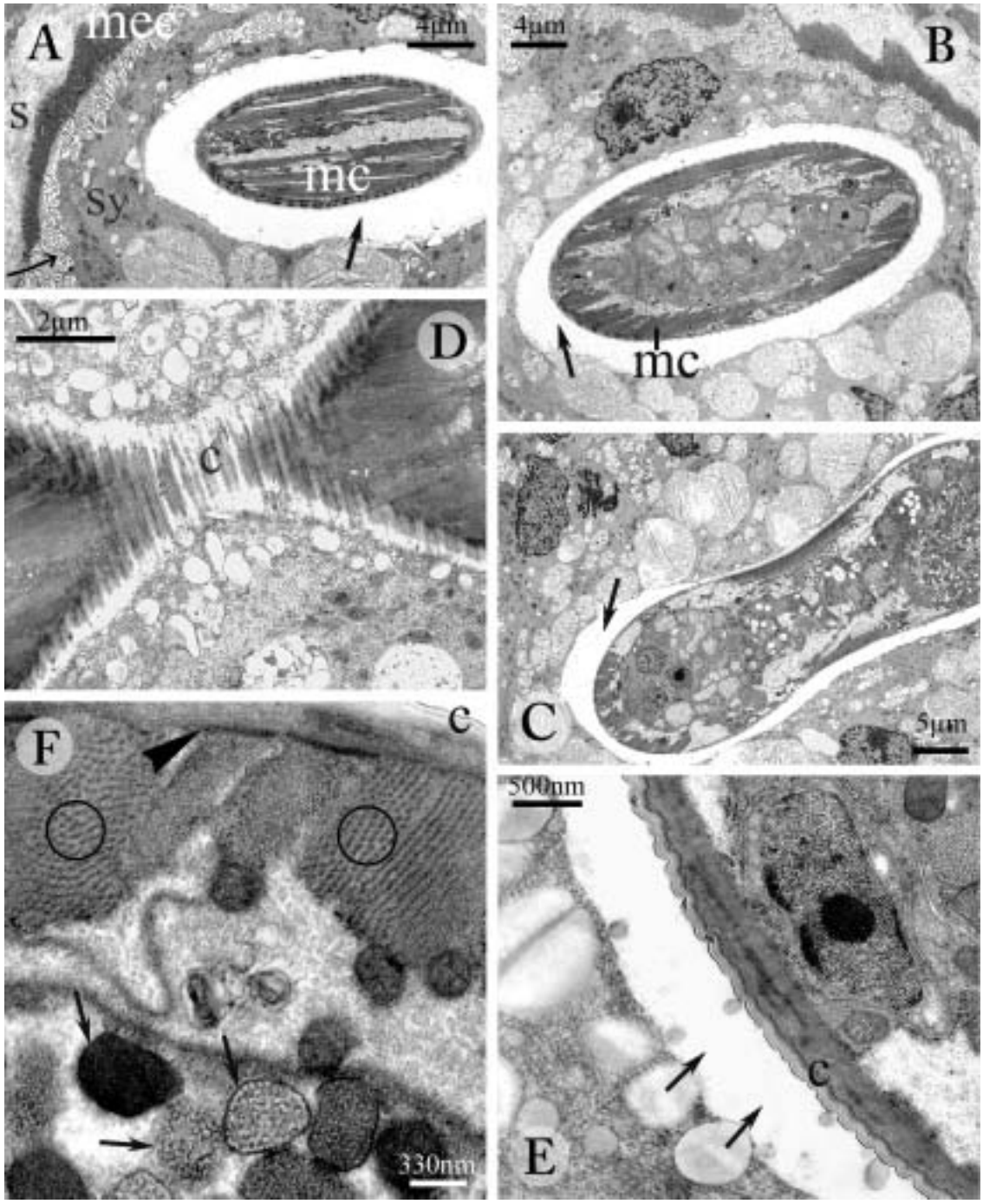


Fig. 3 — Lipid gland of *Phyllomedusa hypochondrialis*, serial sections of a nematode (A-D) and details of its body wall (E, F). A: Tangential section of the worm beneath the hypodermal layer, showing the parallel arrangement of the peripheral muscle cells (mc). Notice secretory release involving large lipid aggregation (straight arrow). A thick network (bowed arrow) of slender cytoplasmic processes occupies the contractile-secretory interstice, clearly distinguishable from the fibre-rich gland stroma (s). These processes emanate both from myoepithelial cells (mec) and secretory syncytium (sy). B: Sequential section of the above nematode, showing its body wall just beneath peripheral muscle cells (mc). As above, arrow points to secretory release of lipid product. C and D: Biconcave and sand-glass profiles suggest that these serial sections correspond to the "elbow" region of an elongated, bowed body. Notice release processes (arrow) in C and annulate cuticle (c) in D. E: Merocrine release of lipid droplets (arrows); notice the sculptured cuticle (c) adhering to a hypodermal cell. F Peripheral muscle cells of the nematode: notice regularly arranged (thin and thick) contractile filaments (circles) and cuticle (c). Arrowhead points to a dense plaque where thin filaments converge, arrows to granules of variable density in a cell contiguous to the peripheral contractile layer.

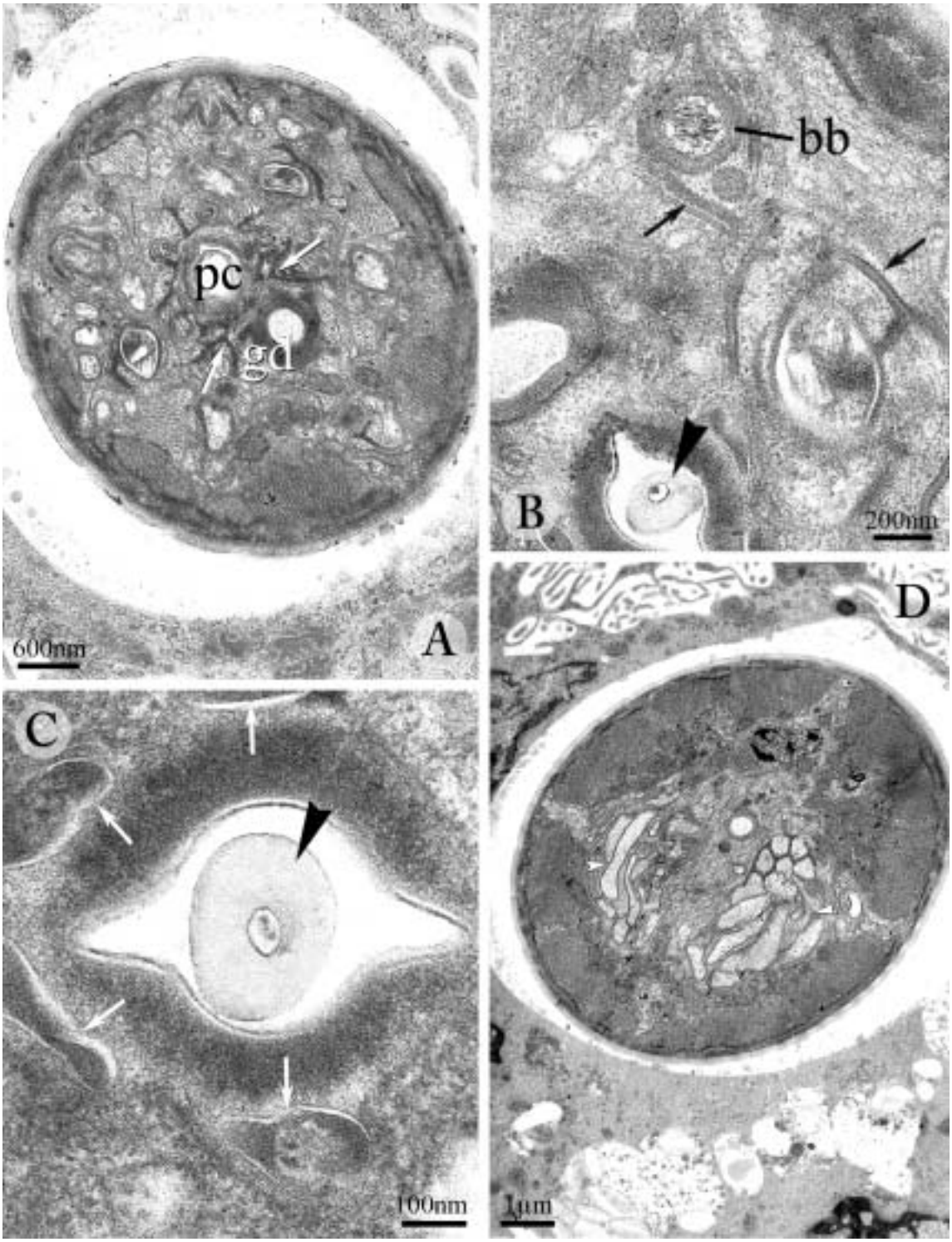


Fig. 4 — The same gland type as above: transversal sections at cephalic and sub-cephalic levels. A: This rostral section shows two cuticular channels: they belong to the oesophageal *procorpus* (pc) and parallel duct of a pharingeal gland (gd), respectively. Arrows point to junctional complexes between oesophageal wall cells. B: Junctional complexes (arrows) also involve dendritic processes from receptor cells provided with modified basal bodies (bb). Arrowhead points to the internal pipe of the gland duct "end apparatus". C: Detail of the pharingeal gland end apparatus. As above, the terminal tract of the gland duct exhibits accessory, cuticular structures: an internal, thick walled channel (arrowhead), and discontinuous external reinforcement (arrows). D: At a more caudal level, bundled axons are obvious (arrowheads), which correspond to paired nerve cords.

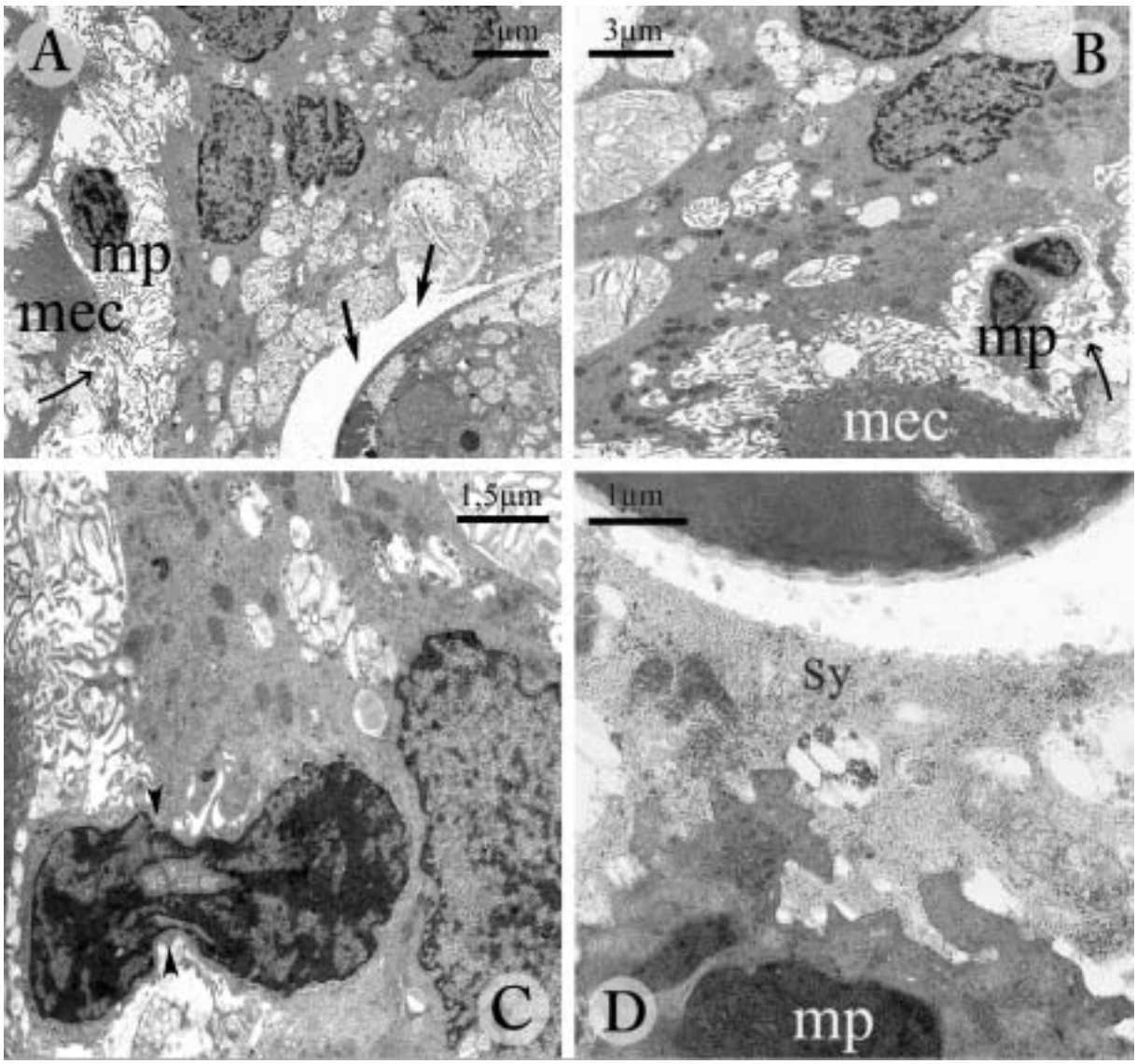


Fig. 5 — Lipid gland of *Phyllomedusa hypochondrialis*: migrating cell responses against nematodes. A: Macrophage (mp) within the contractile–secretory compartment; straight arrows point to secretory release processes into the compartment holding the guest; bowed arrow indicates a gap between myoepithelial cells (mec). B and C: Migrating cells (mp in B) exhibit bilobate nuclei, possibly related to the capability of adapting their shapes during migration (arrowheads in C); as in A, bowed arrow in B indicates a myoepithelium (mec) gap. D: Migrating cell (mp) closely contiguous to a nematode, with interposition of secretory syncytium cytoplasm (sy).

described different amphibian-nematode assemblages, from different continents, the host glands display similar response repertories. Since our study aimed to exploit this serendipity to contribute knowledge on serous gland functional morphology, we have not dwelt on detailed taxonomic determination of the round worms. However, TEM features ascribe the hosted nematodes to the Chromadoria or Rhabditia subclasses, as these include species provided with annulate cuticular sheaths (Malakhov 1994). The foregut region of nematodes we found in the ventral skin glands of *Phyllomedusa hypochondrialis* exhibit ultrastructural traits very similar to those of plant parasites in the subclass Rhabditia (order Tylenchida): *Tylenchorhynchus dubius* (Anderson and Byers 1975), *Heterodera glycines* (Endo 1998). Whereas we cannot exclude that the tree-frogs occasionally pick up round worms from plants, the high numbers of nematodes found in *Rana camerani* suggest that in this species serous glands are elective sites of nematode life cycle, confirmed by the consistent patterns of moulting processes which accompany body growth.

Be these nematodes occasional or usual guests, they do not cause any degenerative change in the glands and therefore they should be regarded as commensal organisms. On the other hand, they are still suitable, natural tracers for studying the inner space system of serous glands, both at the periphery and central region of the secretory syncytium. The concept of a serous syncytium, developed by FARAGGIANA (1938b), implies that there is no interstitial space system within the secretory unit, which lacks any central lumen (GILLOIS-CHEVALIER 1960; DOCKRAY and HOPKINS 1975). Our present findings confirm that there are no intercellular spaces in anuran secretory units, whereas the inner space that under LM looks like a wide lumen (MILLS and PRUM 1984), actually corresponds to the internal portion of the syncytium, not adequately preserved by routine techniques (DELFINO *et al*. 2002). On the contrary, the small cavity beneath the intercalary tract is a proper, albeit exiguous, gland lumen, since it holds secretory material released by the syncytium (DELFINO *et al*. 1994; 1996; 1999b). The consistent patterns of secretory release into the compartments holding the round worms suggests that they are branches of the sub-intercalar gland lumen. The amounts of product released by glands hosting nematodes certainly far exceed the scanty emission observed under resting (constitutive) conditions (DELFINO *et al.* 1994; 1996; 1999b). It appears that the secretory patterns detected in our study are inducible responses against intruder organisms, although they were not so massive as the bulk discharge evoked by adrenergic stimulation of the myoepithelium (DELFINO 1980; MELIS *et al.* 2000; NOSI *et al.* 2002).

Neither in *Phyllomedusa hypochondrialis* nor in *Rana camerani*, secretory activities of serous host glands seem to affect round worms, which posses a complex cuticle sheath. On the other hand, cutaneous lipids of *Phyllomedusa* are harmless molecules which regulate water loss (BLAYLOCK *et al.* 1976), whereas the cutaneous serous products of *Rana* are mostly active against prokaryotic cells (BARTECZKO and KUZIEMSKI 1970; ÇEVIKBAS 1977). Paradoxically, merocrine activity accommodates the nematodes, since the limiting membranes of the secretory granules merge in the plasmalemma and increase the periluminal surface area patch by patch. These processes contribute to the formation of virtual luminal ingrowths with whorl-like and stacked profiles, which in turn lead to a widening of the exiguous, sub-intercalar gland lumen.

The lumen of the duct-neck complex offers a direct way of access to the gland for nematodes coming from the outside, since only a layer of cells separates this cavity from the secretory syncytium (DELFINO *et al.* 1996, on *Rana iberica* and *R. esculenta* complex; NOSI *et al.* 2002, on *Phyllomedusa hypochondrialis*). Alternatively, the nematodes could reach the syncytium from inside, via the stromal environment, by crossing the myoepithelium, that in lipid producing glands exhibits several gaps (NOSI *et al.* 2002). However, this inside pathway does not seem realistic, since we never observed damaged tissues around the glands. On the other hand, macrophages use this path to reach the upper gland levels (DELFINO *et al.* 1998), and enhance their flow against the intruding worms. However, cell migration is an usual, immune response from the stromal micro-environment, whereas the inducible merocrine release and the structural plasticity of the periluminal, plasma membrane are peculiar functional traits in serous glands hosting nematodes.

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