

The subepithelial gland in ants: a novel exocrine gland closely associated with the cuticle surface

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Abstract

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Two glandular systems were discovered that secrete their products onto the cuticular surface in ants. The first, the subepithelial gland, was previously undescribed in ants, and is found throughout the body just beneath the epithelium. This gland consists of independent secretory units, each made up of a single gland cell and an associated duct cell that penetrates the cuticle. Its ultrastructural appearance is consistent with possible hydrocarbon production. Examining 84 ant species, the subepithelial gland was found in eight subfamilies (out of 13), although not necessarily in all species. In a single ant species, *Harpegnathos saltator*, it was the epithelium itself that was enlarged and functioned as a gland. The enlarged epithelial cells secrete their products directly onto the cuticle through distinct cuticular crevasses.

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Introduction

The cuticle is an essential structure for the survival and success of terrestrial insects. The inert layer of protein and chitin serves as a mechanical barrier and as a strong but light exoskeleton for body rigidity and muscle attachment. The outermost part of the cuticle, the epicuticle and associated wax layers, functions as a chemical barrier, essential in restricting the loss of water from the body and the penetration of microorganisms (Gullan and Cranston 1994). Hydrocarbons on the cuticle are produced in oenocytes and epidermal cells and are subsequently transported to the epicuticle (Lockey 1988; Gu *et al.* 1995; Young *et al.* 1999). As the cuticle is the first contact point between individuals, chemicals on the surface of the cuticle often play a role in communication. This is especially important in social insects, where complex societies require constant interactions between nestmates.

The cuticular chemistry of social insects is mainly involved in interindividual recognition. Kin recognition cues, which ensure colony integrity through discrimination against foreign ants (Breed and Bennett 1987; Crosland 1989a,b; Breed 1998; Lahav *et al.* 1999; Zanetti *et al.* 2002), are acquired in part from a colony's specific environment, adding up to the hydrocarbons each individual produces itself. Apart

from colony membership, cuticular hydrocarbons can convey accurate information about an individual's physiological state. Quantitative or qualitative differences in hydrocarbon profiles of fertile and sterile individuals allow recognition of a nestmate's reproductive status or age (Monnin *et al.* 1998; Liebig *et al.* 2000; Cuvillier-Hot *et al.* 2001; Sledge *et al.* 2001). A cuticle's chemical signature is thus flexible and can change rapidly according to an individual's status.

Though about 100 exocrine glands are known in social insects (Billen 2002), the exact origin of the pheromones on the cuticle remains poorly understood. In bumble bees, similar hydrocarbon compounds to the cuticle are found in the Dufour's gland (Oldham *et al.* 1994). In ants, the contents of the postpharyngeal gland resemble the hydrocarbons on the cuticle (Bagnères and Morgan 1991; Soroker *et al.* 1995). Recent evidence suggests a sequestering rather than a secretory function for this gland, as ants with the postpharyngeal glands removed retain their cuticular hydrocarbon profile (Soroker *et al.* 1994). In the termite *Kalotermes flavicollis*, singular secretory cells occur among the integumental dermal cells and secrete their contents onto the cuticular surface (Sbrenna and Leis 1983). These authors suggested a possible pheromonal function of the secretion as the ultrastructural appearance of this gland changes with age.

This study describes a gland closely associated with the tegumental cuticle that is novel in ants. This gland releases its contents through the outer surface of the cuticle, possibly affecting the chemical composition of the ant's integument although its exact function remains elusive. The structure of this gland and its occurrence throughout the ant subfamilies are discussed to encourage further research into its function in particular species.

Methods

We investigated the occurrence of the subepithelial gland in 84 ant species of the 13 major ant subfamilies. The investigated specimens were collected at various localities (see Tables 1 and 2) and transported to Belgium where heads, and occasionally thoraxes and abdomens, were prepared for electron microscopy. Observations were concentrated on the posterior dorsal part of the head, as this region contains no pores of other known exocrine glands. For scanning electron microscopy (SEM), specimens were air-dried and subsequently coated with gold (5 nm) and viewed in a Philips XL30 ESEM microscope. Dirty specimens were rejected to avoid false negatives. For transmission electron microscopy (TEM) heads were cut open frontally for better fixation, and were fixed for 24 h in 2% glutaraldehyde (4 °C, pH 7.3 and buffered with 0.05 M sodium cacodylate). Postfixation in a buffered osmium tetroxide solution (1 h) was followed by dehydration in an acetone series, embedding in Araldite, and sectioning with a Reichert Ultracut E ultramicrotome. To check fixation quality under light microscopy, semi-thin sections (1 µm) were stained with methylene blue and thionin. Thin sections for TEM (70 nm) were double-stained with uranyl acetate and lead citrate in an LKB 2168 Ultrastainer. The ultrastructural organization of the subepithelial glands was examined with a Zeiss EM 900 microscope. Some negative SEM samples were double-checked using TEM to confirm the absence of the subepithelial gland.

Results

The subepithelial gland consisted of scattered glandular secretory units situated just below the thin epidermis (1 µm) lining the ant's exoskeleton. A glandular unit consisted typically of a single secretory cell and a single duct cell. The secretory cells had a diameter of about 13 µm (Fig. 1A). Each cell had an active nucleus of about 7-µm diameter. Mitochondria were cylinder-shaped, with a diameter of 0.6 µm, while in some cells there were giant mitochondria of about 2.5 µm × 4.5 µm. The cells contained clusters of smooth endoplasmic reticulum (Fig. 1B), suggesting production of a nonproteinaceous secretion product. The cytoplasm contained a Golgi apparatus, numerous free ribosomes, and occasional microtubules. The secretory cell discharged its contents through the end apparatus into the duct cell. The microvilli of the end apparatus were often distorted, suggesting

Table 1 The occurrence of the subepithelial gland in 44 species of five tribes of the ant subfamily Ponerinae

Species	Origin	Subepithelial gland
Ponerinae		
Ectatommini		
<i>Gnamptogenys bicolor</i>	Indonesia	+
<i>Gnamptogenys binghamii</i>	Indonesia	+
<i>Gnamptogenys costata</i>	Indonesia	+
<i>Gnamptogenys dammermani</i> ¹	Indonesia	+
<i>Gnamptogenys menadensis</i> ^{1,2,3}	Brazil	+
<i>Gnamptogenys moelleri</i>	Indonesia	+
<i>Gnamptogenys laevior</i>	Malaysia	+
<i>Gnamptogenys</i> sp. M12	Malaysia	+
<i>Gnamptogenys</i> sp. M6	Malaysia	+
<i>Gnamptogenys posteropsis</i> ²	Malaysia	+
<i>Gnamptogenys</i> sp. M9	Costa Rica	+
<i>Gnamptogenys striatula</i>	Costa Rica	+
<i>Gnamptogenys porcata</i>	Costa Rica	+
<i>Gnamptogenys tornata</i>	Australia	+
<i>Rhytidoponera aurata</i>	Australia	+
<i>Rhytidoponera</i> sp. B	Mexico	+
<i>Ectatomma ruidum</i>	Mexico	+
<i>Ectatomma opaciventris</i>		
Amblyoponini		
<i>Amblyopone reclinata</i>	Indonesia	–
<i>Myopopone castanea</i>	Indonesia	+
<i>Mystrium camilae</i>	Indonesia	–
Ponerini		
<i>Anochetus</i> sp. 2	Indonesia	–
<i>Centromyrmex feae</i>	Indonesia	+
<i>Diacamma</i> sp. Japan ¹	Japan	+
<i>Dinoponera quadriceps</i>	Brazil	+
<i>Harpegnathos saltator</i> ^{1,2*}	India	–
<i>Myopias maligna</i>	Indonesia	+
<i>Myopias emeryi</i>	Indonesia	+
<i>Leptogenys myops</i> ⁴	Indonesia	+
<i>Leptogenys diminuta</i>	Indonesia	–
<i>Odontomachus rixosus</i>	Indonesia	–
<i>Odontomachus simillimus</i>	Indonesia	–
<i>Pachycondyla astuta</i>	Indonesia	–
<i>Pachycondyla leeuwenhoekii</i>	Indonesia	–
<i>Pachycondyla obscuricornis</i>	Costa Rica	–
<i>Pachycondyla (Bothroponera)</i> sp.	Indonesia	–
<i>Plectroctena</i> sp.	Burundi	+
<i>Prionopelta kraepelini</i>	Indonesia	–
<i>Streblognathus peetersii</i> †	Very Coast	+
Plathythyreini		
<i>Plathythyrea</i> sp. 5	Indonesia	–
<i>Plathythyrea punctata</i>	Puerto Rico	–
<i>Probolomyrmex dammermani</i>	Indonesia	–
Proceratiini		
<i>Discothyrea</i> sp.	Japan	–
<i>Proceratium itoi</i>	Japan	–

As default, the back of the head of workers of each species was examined with scanning electron microscopy. Deviations from this protocol are indicated by:

¹ scanning and transmission electron microscopy; ² head, thorax and abdomen; ³ queen, worker and male; ⁴ queen.

* Special epithelium; † thorax; Virginie Cuvillier-Hot (personal communication).

Table 2 The occurrence of the subepithelial gland in 40 species of 12 ant subfamilies, using the same protocol as in Table 1

Species	Origin	Subepithelial gland	Species	Origin	Subepithelial gland
Aenictinae			Myrmeciinae		
<i>Aenictus rotundatus</i> ¹	Kenya	+	<i>Myrmecia gulosa</i>	Australia	+
Aneurtinae			<i>Myrmecia pyriformis</i>	Australia	+
<i>Aneurtus simone</i> ¹	Sri Lanka	–	<i>Myrmecia simillima</i>	Australia	–
Cerapachyinae			<i>Myrmecia vindex</i>	Australia	–
Cerapachyini			Myrmicinae		
<i>Cerapachys jacobsoni</i>	Indonesia	+	Attini		
<i>Cerapachys suscitatus</i> ¹	Indonesia	+	<i>Acromyrmex octospinosus</i>	Panama	–
Cylindromyrmecini			Cephalotini		
<i>Cylindromyrmex whympersi</i> ²	Costa Rica	+	<i>Cephalotus umbraculatus</i>	Costa Rica	+
Dolichoderinae			Dacetini		
<i>Azteca near bicolor</i> ^{1,2}	Brazil	–	<i>Strumigenys rogeri</i>	Indonesia	–
Dorylinae			<i>Strumigenys</i> sp. ^{2,4}	Costa Rica	–
<i>Dorylus molestus</i> ⁵	Kenya	–	<i>Strumigenys lewisi</i>	Japan	–
Ecitoninae			Formicoxenini		
<i>Eciton burchelli</i> ¹	Brazil	–	<i>Leptothorax acervorum</i>	Belgium	+
<i>Labidus praedator</i>	Brazil	–	Myrmecini		
Formicinae			<i>Acanthomyrmex minus</i>	Indonesia	–
Camponotini			<i>Acanthomyrmex padaniensis</i>	Indonesia	–
<i>Camponotus floridanus</i>	USA	–	<i>Acanthomyrmex sulawesiensis</i>	Indonesia	–
<i>Camponotus zulu</i>	Kenya	–	<i>Acanthomyrmex ferox</i> ⁴	Malaysia	–
Formicini			Myrmicini		
<i>Cataglyphis iberica</i>	Spain	–	<i>Myrmica rubra</i>	Belgium	–
<i>Cataglyphis cursor</i>	France	–	Pheidolini		
<i>Formica fusca</i> ¹	Belgium	–	<i>Messor bouvieri</i>	Italy	+
<i>Formica pratensis</i>	Belgium	–	<i>Pheidole fervida</i> ⁵	Japan	–
<i>Rossomyrmex minuchae</i>	Spain	–	Solenopsidini		
Lasiini			<i>Solenopsis aurea</i>	USA	–
<i>Lasius fuliginosus</i>	Belgium	+	<i>Solenopsis invicta</i> ⁶	USA	–
Oecophyllini			Nothomyrmecinae		
<i>Oecophylla longinoda</i>	Kenya	–	<i>Nothomyrmecia macrops</i>	Australia	+
Leptanillinae			Pseudomyrmecinae		
Leptanillini			<i>Pseudomyrmex</i> sp.	Mexico	+
<i>Leptanilla clypeata</i>	Indonesia	–			

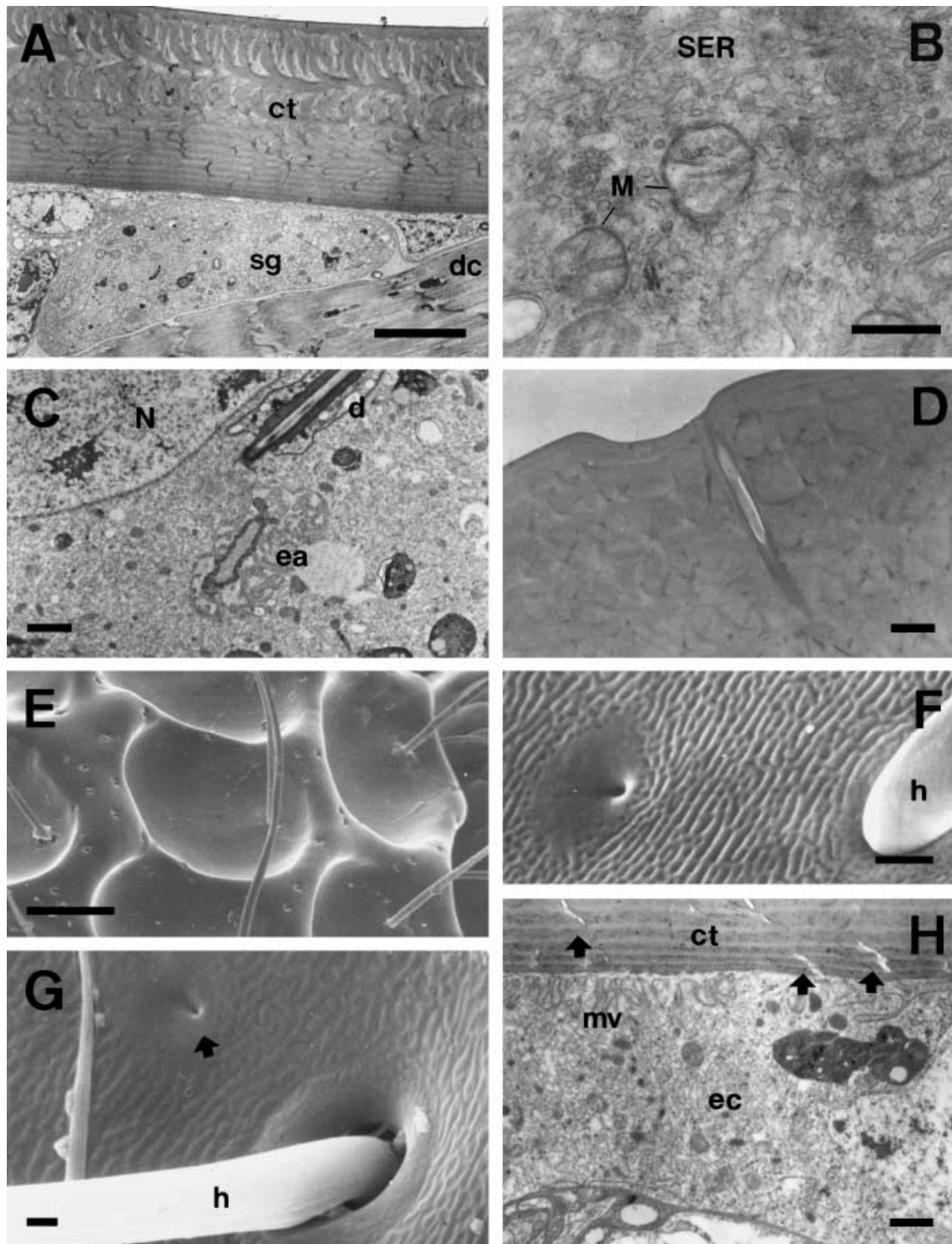
As default, the back of the head of workers of each species was examined with scanning electron microscopy. Deviations from this protocol are indicated by:

¹ scanning and transmission electron microscopy; ² head, thorax and abdomen; ³ queen, worker and male; ⁴ queen; ⁵ soldier and worker; ⁶ queen and male.

substantial secretory activity. Ducts had an internal diameter of 0.3–0.4 μm and were lined with a 0.1- μm -thick layer of cuticle (Fig. 1C,D). The duct penetrated the epidermis and exoskeleton of the ant to open at the outside surface through a pore with a diameter of 0.1–0.5 μm (Fig. 1E, F,G). In most species, the cuticular opening had a widened appearance, forming a small cup or a small trench. Only a few species (*Centromyrmex* sp., *Diacamma* sp., *Dinoponera quadriceps*, *Lasius fuliginosus*) lacked this modification and had smaller apertures, similar in diameter to a typical gland duct. The gland pores were erratically distributed over the body surface, with a density of up to 8 per 100 μm^2 in some species (Fig. 1E). When thoraxes and abdomens were available from species that had the gland in their head, these also showed pores and glands (Tables 1 and 2). The different glandular units thus formed an extensive secretory system covering the entire body.

The subepithelial gland was found in species belonging to eight out of 13 ant subfamilies. In the subfamily Ponerinae the gland occurred in species from three out of five common tribes (Table 1). It occurred in all species of three investigated genera from the ponerine tribe Ectatommini (Table 1), comprising 14 species of *Gnamptogenys*, originating from both the Neotropics and Indo-Malayan regions. In contrast, the gland was not always present in other ponerine tribes. The subepithelial gland occurred in eight species (out of 18 investigated) in the tribe Ponerini and in one species (out of three) in the tribe Amblyoponini. (Table 1).

In the ant subfamily Cerapachyinae, the subepithelial gland occurred in all three investigated species from two tribes (Table 2). *Nothomyrmecia macrops*, the sole species in the subfamily Nothomyrmecinae, also showed conspicuous subepithelial gland pores. In the subfamily Myrmeciinae, two species showed the typical pores in high density, but no pores



could be detected in two other species (Table 2). The gland was present in each of a single investigated species in the subfamilies Aenictinae and Pseudomyrmecinae. In the subfamilies Formicinae and Myrmicinae, the occurrence of the subepithelial gland was more erratic. Only one out of nine Formicinae (from four tribes) and three out of 14 Myrmicinae species (three out of eight tribes) had the gland.

One more exceptional glandular specialization associated with the cuticle was found exclusive to the ponerine ant species *Harpegnathos saltator*. Here, workers have no subepithelial gland, but instead some parts of the epidermis contained 10-fold broadened cells (6–8 µm) with abundant inclusions and dense microvilli (Fig. 1H). Numerous cracks in the cuticle, found only opposite the broadened epidermal cells, might facilitate the discharge of secretion through the cuticle. As no ducts were associated with the epithelium, scanning electron micrographs of the cuticle did not reveal round pores as with the subepithelial gland.

Discussion

Numerous subepithelial gland units form a dense secretory system covering the entire ant's body. The glands are not associated with joints or intersegmental membranes, but are more or less regularly distributed underneath the epidermis. Each independent glandular unit consists of a single secretory cell and its associated duct cell, which allows categorization as type 3 glands in Noirot and Quennedey's (1974) classification. The structure and location of subepithelial glands in ants somewhat resembles that of the integumental glands described in termites (Leis and Sbrenna 1983; Sbrenna and Leis 1983). However, those integumental glands occur in dense patterns with multiple secretory cells touching each other.

Each glandular unit of the subepithelial gland secretes its contents directly onto the surface of the cuticle. The simple ducts serve for transport only and have no storage function as they are not widened (see Gobin *et al.* 2001). The presence of a smooth endoplasmic reticulum in the gland cells suggests that the secretion does not contain proteins, but is in concordance with a possible production of hydrocarbons. Although the exact nature of the secretion is not

known, products from the subepithelial gland must modify the chemical constitution on the cuticular surface. The basic hydrocarbon profile of ants probably arises from oenocyte-derived products as in solitary insects (Young *et al.* 1999). However, by adding substances to the resident cuticular hydrocarbons, the subepithelial gland could induce fast changes in the chemical profile of the cuticle, necessary for communication. Workers from four species that have the gland are known to use direct cuticular contact for status recognition: *Gnamptogenys menadensis*, *Diacamma* sp. from Japan, *Dinoponera quadriceps* and *Streblognathus peetersi* (Gobin *et al.* 1999; Monnin and Peeters 1999; Tsuji *et al.* 1999; Cuvillier-Hot *et al.* 2002b). Indeed, chemical profiles of the cuticle can give unequivocal information on an individual's fertility (Peeters *et al.* 1999; Cuvillier-Hot *et al.* 2002a). *Harpegnathos saltator* also has status identification depending on cuticular chemistry (Liebig *et al.* 2000), but lacks the subepithelial gland. However, workers of this species have an exceptional glandular epidermis that apparently secretes its product through crevasses in the cuticle. A similar highly developed epithelium is probably the source of an attractant signal in queens of the ponerine ant *Megaponera foetens* (Hölldobler *et al.* 1994). Such glandular epithelium is a distinct gland type (type 1) from the subepithelial gland (type 3) in Noirot and Quennedey's (1974) classification.

Knowledge of the exact function of cuticular chemicals is nevertheless limited for most ant species. We should thus be careful in our interpretation of a function of the subepithelial gland, avoiding exclusion of possible roles in desiccation prevention and kin recognition. In ants, the same gland can indeed have diverse biological functions in different subfamilies (Billen and Morgan 1998). Though present in eight of the 13 investigated ant subfamilies, it is especially common in a single ponerine tribe Ectatommini, suggesting phylogenetic inertia in its prevalence, perhaps linked to a specific function. Conceivably the erratic occurrence of the subepithelial gland across ant subfamilies will allow for future testing of specific functional hypotheses through comparison of species that have, or lack, the gland. Unfortunately, the small size of gland cells and their scattered occurrence in the ant's body hamper the use of bio-assays aimed at elucidating the subepithelial gland's function.

Fig. 1—**A.** Transmission electron micrograph (TEM) of the subepithelial gland in *Aenictus rotundatus* (scale bar = 5 µm). sg = subepithelial gland cell; dc = duct cell; ct = cuticle. —**B.** Detail of the cytoplasm of *Pseudomyrmex* sp. showing smooth endoplasmic reticulum (SER), mitochondria (M) and scattered free ribosomes (scale bar = 0.5 µm). —**C.** TEM of a subepithelial gland cell in *Gnamptogenys menadensis*. The secretory cell shows an end apparatus (ea) through which secretory products are transported into a cuticle-lined duct (d), and the nucleus (N) (scale bar = 1 µm). —**D.** TEM of duct (d) penetrating cuticle in *G. menadensis*. The cup-like impression in the surface is clearly visible (scale bar = 1 µm).

—**E.** Scanning electron micrograph (SEM) of a *Gnamptogenys posteropsis* head showing abundant pore openings of subepithelial glands (scale bar = 50 µm). —**F.** SEM of *Myopias emeryi* head with detail of the modification of the cuticle structure around the duct opening; h = hair (scale bar = 1 µm). —**G.** SEM detail of the gland opening (arrow) in the head cuticle of 'living fossil' *Nothomyrmecia macrops*; h = hair (scale bar = 1 µm). —**H.** TEM of the broadened epithelial cells (ec) in *Harpegnathos saltator* showing numerous microvilli (mv). Typical cracks (arrows) in the cuticle (c) are only found adjacent to widened epithelial cells and might facilitate secretion (scale bar = 1 µm).

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