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THE SPIRACLE GLANDS IN *IXODES RICINUS* (LINNAEUS, 1758) (ACARI: IXODIDAE)

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ABSTRACT — Within Ixodida, dermal glands associated with spiracles are apparently an exclusive feature of Ixodidae. The function of these glands is still unclear and morphological studies revealed differences between species. The structure of the gland in *Ixodes ricinus* was examined using electron microscopy. The results are compared with the findings in previously studied species and possible functions of the glands are discussed.

KEYWORDS — spiracle; basal plate glands; calyx; sclerites; hygroscopic secretion; pheromones

INTRODUCTION

Ticks or Ixodida include the families Nuttalliellidae Schulze, 1935, Argasidae Canestrini, 1890 and Ixodidae Murray, 1887. Within the Ixodidae, two major groups - Ixodinae and Amblyomminae - are distinguished. The Ixodinae include all species of the genus *Ixodes*, and the Amblyomminae unite all other genera of the Ixodidae (El Shoura *et al.*, 1984; Filippova, 2008; Oliver, 1989). Depending on the developmental stage (egg, larva, nymph, adult), two different types of respiration are known in ticks. The transport of gases directly through the integument is sufficient in larvae. Increasing cuticle thickness and body size prevent cuticular respiration in nymphal and adult ticks. They use the tracheae, which access ambient air by a pair of complex spiracles (Coons and Alberti, 1999; Pugh, 1997; Sonenshine, 1991).

Spiracle structure and function in ticks has drawn the attention of scientists for more than a century (e.g., Arthur, 1956; Fielden *et al.*, 2011; Hefnawy, 1970; Nordenskiöld, 1906; Woolley, 1972). Spiracles show unique features typical for each of the three different tick families (Latif *et al.*, 2012; Pugh, 1997; Roshdy *et al.*, 1983; Sonenshine, 1991). In ixodid ticks, one spiracle is located ventrally behind the coxa of the fourth leg. Each spiracle consists of a labyrinth of chambers formed by cuticular columns (pedicels) positioned on a thickened baseplate. The pedicels are covered by a sieve-plate with numerous differently sized pores (aeropyles) surrounding the macula, a pore-less area. In adults it contains the sealed "ostium", an ecdysial remnant of the nymphal stage. Air, reaching the labyrinth over the pores, is collected in the subostial space. This space is connected with the tracheal atrium, the origin of the main tracheal trunks. Different kinds of chambers are distinguished in the labyrinth of Ixodidae. One of them, the primary atrial chamber, is connected by a pore to the body surface and via a

cuticular duct to the hypodermis (Pugh *et al.*, 1988; Pugh, 1997).

These cuticular ducts in the baseplate, and the cytoplasmic extensions of the hypodermal layer within them, are typical of spiracles in Ixodidae (Pugh, 1997; Roshdy *et al.*, 1983). In previous descriptions they were interpreted as sensory structures (Nordenskiöld, 1906; Roshdy and Hefnawy, 1973), as "Krobylophoren", organs of sensory and glandular function (Schulze, 1942), or as integumental glands (Arthur, 1956). Scanning and transmission electron microscopy (SEM and TEM) studies revealed more details on spiracle morphology (Hinton, 1967; Woolley, 1972) and supported a glandular nature of the cytoplasmic extensions (Baker, 1997; Sixl and Sixl-Voigt, 1974; Walker *et al.*, 1996). Nevertheless, there are morphological differences in the spiracle glands of the species studied, and their function remains unclear. Pugh (1997) suggested that they produce a hygroscopic substance to reduce water loss during respiration. As they have a connection to the tick's surface over primary atrial chambers, they are also possible sites for the production of semiochemicals (Walker *et al.*, 1996). This large group of information-bearing chemical compounds includes pheromones that are of special interest for tick control: the addition of tick pheromones to acaricides can enhance their effectiveness and enables the development of new control devices (Gachoka *et al.*, 2012; Sonenshine, 2006).

The most abundant tick species in Austria is *Ixodes ricinus*, which inhabits most parts of Europe. It has an extremely broad host spectrum and is one of the relatively few tick species readily feeding on humans. Accordingly, it is the most important vector of the severe human diseases Lyme borreliosis and tick-borne encephalitis in Europe. It is also a competent vector for several other pathogens (Estrada-Pena and Jongejan, 1999; Stanek, 2009). The present study provides new information on the morphology of spiracle glands in *I. ricinus* using SEM- and TEM-techniques and discusses their possible function.

MATERIALS AND METHODS

Unfed specimens of adult ixodid ticks were collected in March 2012 by dragging a white cotton cloth through vegetation of a deciduous forest on the southern border of Vienna. Males and females were separated, and *I. ricinus* was determined under a stereo microscope. Ticks were kept in glass jars with perforated lids for air supply. High air humidity was ensured by moistening paper towels within the containers at regular intervals.

Within one week after collection, the specimens were immerged and dissected in cold fixative (modified Karnovsky: 3 % glutaraldehyde and 2 % paraformaldehyde in 0.1 M phosphate buffer at a pH of 7.2) under a fume hood. The parts of the specimens containing the spiracles were transferred into Eppendorf tubes filled with fresh cold fixative and fixed for 12 h at 4 °C on a shaking plate followed by post-fixation on ice with 2 % osmium tetroxide (OsO_4) in PBS for 2 h after rinsing in phosphate buffer solution (PBS). Then they were dehydrated in a graded series of ethanol and embedded in Epon 812 resin with acetone as intermedium. Sectioning was done with a Reichert Ultracut E. For better orientation within the specimens, semi-thin sections were made, stained with toluidine blue and examined with a Nikon Eclipse E800 light microscope (LM). Ultrathin sections were mounted on Formvar-coated slot grids and stained with uranyl acetate and lead citrate or lead citrate only. Transmission electron microscopy (TEM) was performed with a Zeiss Libra 120 at 120 kV.

For scanning electron microscopy (SEM), whole specimens preserved in 70 % ethanol and razor blade sections through spiracle plates were dried with hexamethyldisilazane. They were then glued to aluminium stubs using carbon plates, coated with gold in an Agar B734 sputter coater, and examined in a Philips REM XL 20 at 15 kV.

RESULTS

The spiracle plates in *I. ricinus* – located ventrally, posterior to the fourth legs – are circular in females (Figures 1a and 1b) and ovoid in males. In females

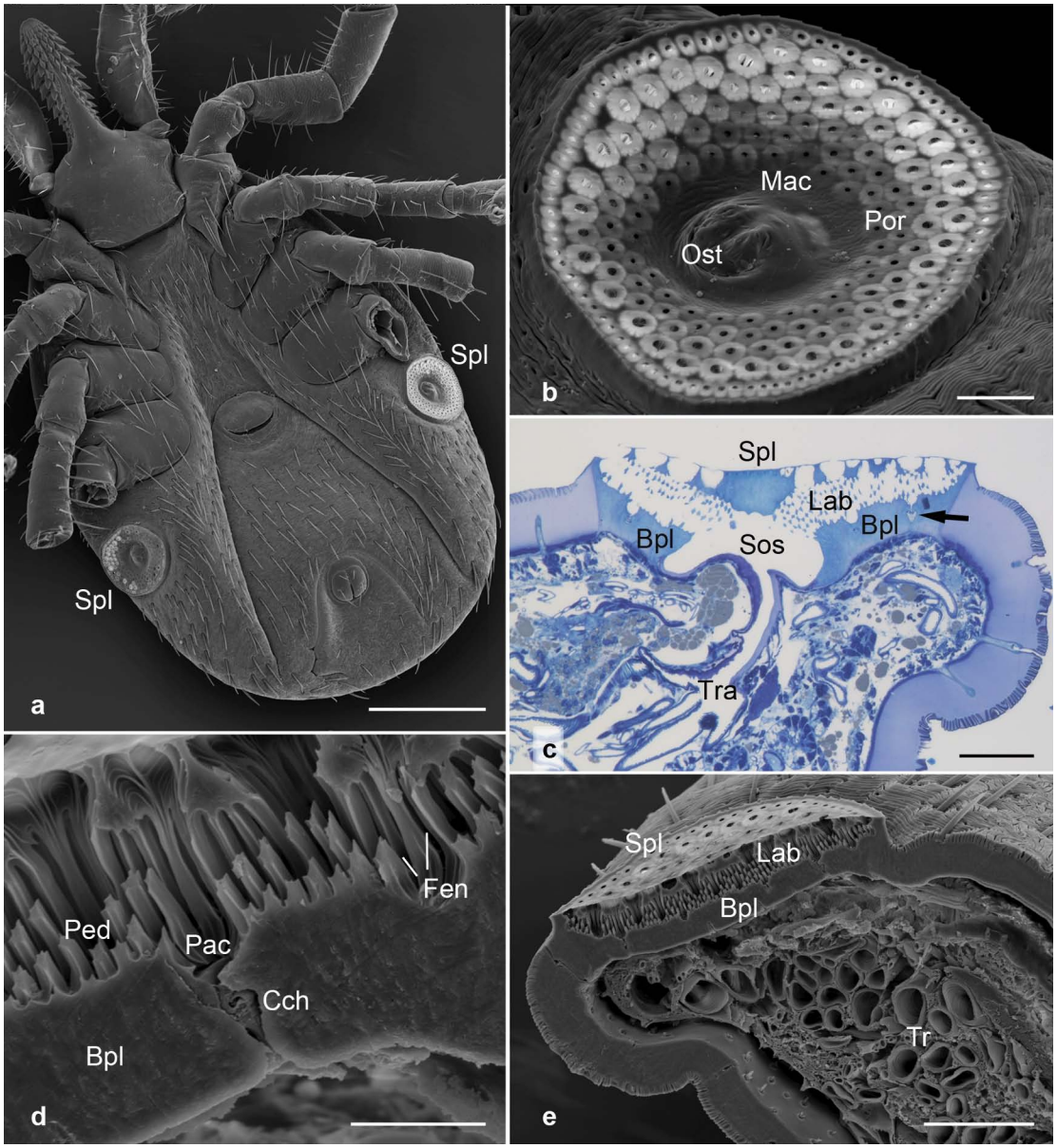


FIGURE 1: Scanning electron microscopy (SEM) and light microscopy (LM) images of female *I. ricinus*. a – Position of the spiracle plates on the ventral side behind coxae IV. (Legs III and IV are partially removed). Scale bar: 500 μm; b – Overview of a spiracle plate with the pore-less area of the macula and the sealed "ostium" in the center. Around the macula numerous radially arranged pores allow gas exchange. Scale bar: 50 μm; c – Semi-thin section (toluidine blue) through a spiracle showing the cuticular channel of the gland cells within the baseplate (arrow). Note the connection of the labyrinth under the spiracle plate with the subostial space and the tracheal atrium. Scale bar: 50 μm; d – Detail of the labyrinth with numerous pedicels positioned on the thick baseplate. Narrow elongated fenestrations connect the chambers within the labyrinth. The cuticular channel in the baseplate leads into a primary atrial chamber. Note that the pedicels are not triangular in cross-section as in Amblyomminae. Scale bar: 20 μm; e – Razor blade section through the porous area of the spiracle plate (sieve-plate) giving an overview of the labyrinth and the baseplate. Numerous cross-sections of tracheal trunks are visible in the tissue below. Scale bar: 100 μm. Bpl, baseplate; Cch, cuticular channel; Fen, fenestrations; Lab, labyrinth; Mac, macula; Ost, sealed "ostium"; Pac, primary atrial chamber; Ped, pedicels; Por, pores; Sos, subostial space; Spl, spiracle plate; Tr, tracheal trunks; Tra, tracheal atrium.

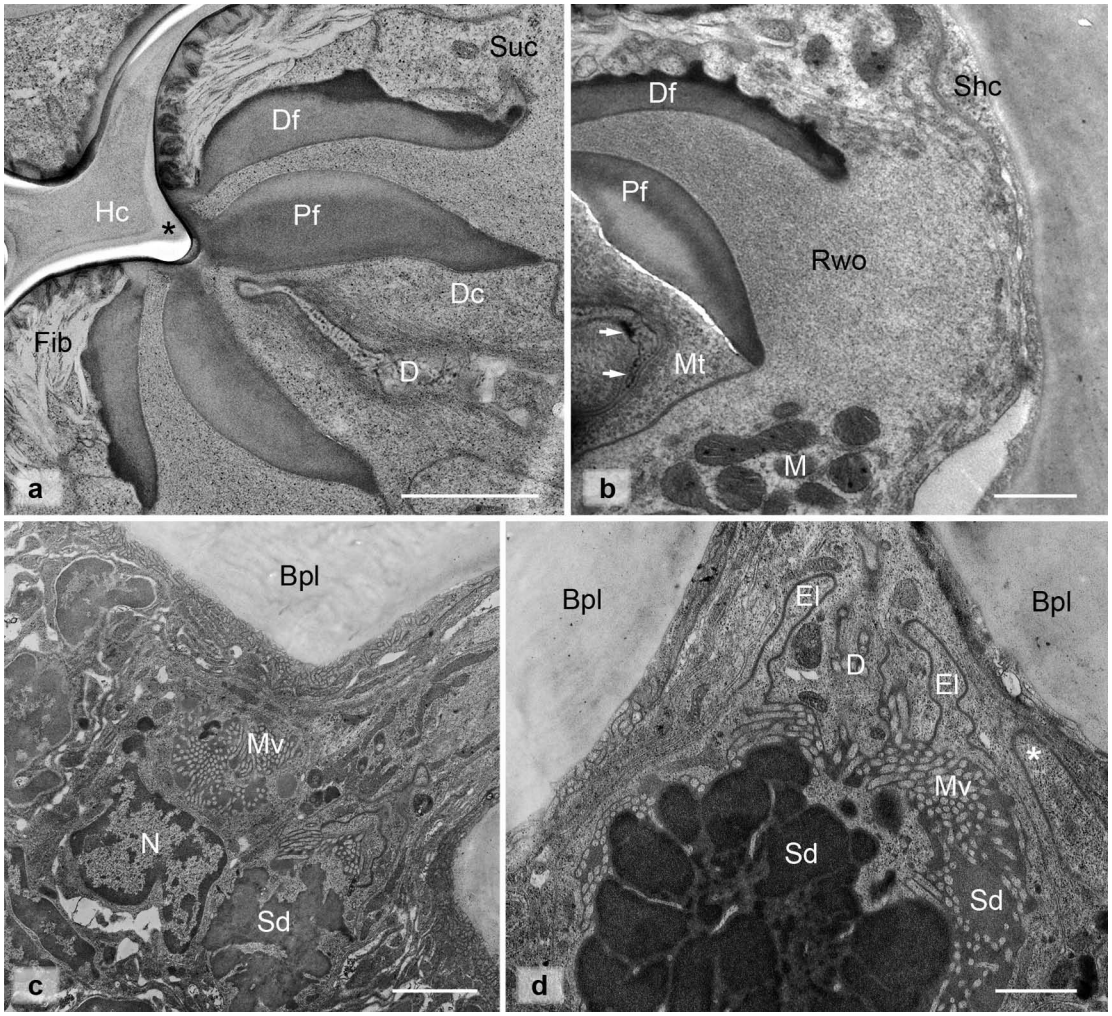


FIGURE 2: TEM images of the spiracle glands in *I. ricinus*. a – Calyx region in a female with the flaps below the horizontal cavity. Fibres are between the distal flaps and the horizontal cavity, containing granular secretion with an electron-lucent "halo" (asterisk). The duct is visible between the proximal flaps. Scale bar: 1 μm; b – Detail of the calyx region in a male (stained with lead citrate only) showing the homogeneous region without cell organelles between the flaps. The duct supported by microtubules contains fibrous structures (arrows). Scale bar: 0.5 μm; c – Overview of the hypodermal layer underneath the baseplate with two secretory cells below the cuticular channel of the baseplate. In the depressions formed by the secretory cells numerous microvilli and electron-dense secretory deposits are obvious. Multiple image alignment; Scale bar: 2 μm; d – Neck of a secretory cell visible as elongations at the base of the cuticular channel. Between them microvilli of the cup-shaped depression reach into the duct. Note the projection (asterisk) indicating the neck of the second secretory cell on the right side of the picture and the different electron-density of the secretory material. Multiple image alignment; Scale bar: 1 μm. Bpl, baseplate; D, duct; Dc, duct cell; Df, distal flap; El, elongations of the secretory cell neck; Fib, fibres; Hc, horizontal cavity; M, mitochondria; Mt, microtubules; Mv, microvilli; N, nucleus; Pf, proximal flap; Rwo, region without cell organelles; Sd, secretory deposits; Shc, sheath cell; Suc, support cell.

the eccentric depressed macula in the slightly elevated plate is encircled by rows of pores. The pore diameter decreases towards the centre of the plate. An additional row of small peripheral pores is present at the periphery of the plate. The "ostium" in the pore-less macula consists of two protrusions and a semi-circular furrow (Figure 1b). Pedicels with lateral processes (Figure 1d) form different-sized chambers within the labyrinth. Narrow, elongated fenestrations between the pedicels connect the chambers (Figure 1d). Pedicels are positioned on the thick cuticular baseplate, which is perforated by connections to the hypodermis (Figures 1c and 1d). The labyrinth is connected to the sub-ostial space and the tracheal atrium (Figure 1c). Numerous cross-sections of tracheae are visible below the spiracle plate (Figure 1e).

The spiracle glands in adult *I. ricinus* are located under the baseplate and consist of four different cell types: two secretory cells, one duct cell, one support cell and two sheath cells. There are two bulbous secretory cells, each forming a cup-shaped depression lined by numerous microvilli (Figures 2c and 2d). The secretory cells contain basal, elongated nuclei, low amounts of rough endoplasmatic reticulum, Golgi bodies, vesicles with contents similar to those in the duct lumen, and numerous mitochondria. Large numbers of differently sized vesicles aggregate near the cell membrane of the depressions. Some of the vesicles seem to aggregate with each other. Densely arranged microtubules are predominant in the duct cell around the lumen. Each secretory cell elongates into a neck (Figure 2d), which unites with the duct leading to the horizontal cavity. The cell forming the duct is tightly fixed to the secretory cells by septate desmosomes (Figure 3a). In the distal part of the cuticular channel the duct (Figures 3b and 3c) initially widens, then narrows (Figure 2a) and opens into the wide cuticular horizontal cavity. The latter is connected to the primary atrial chamber of the labyrinth. Two pairs of cuticular flaps, a proximal and a distal one, are linked to the horizontal cavity. The duct cell is delimited by the insides of the proximal flaps (Figure 2a). A third cell type, the support cell of the gland, extends from the hypodermal layer to the upper surface of the distal flaps. It surrounds the duct cell completely in the area below the horizontal cavity (Figure 3c). Numerous microtubuli and large mitochondria are conspicuous within the support cell. The distal pair of flaps is often electron-dense on the sides facing the horizontal cavity and form irregular projections (Figures 2a and 2b). Similar structures surround the horizontal cavity and its connection to the chamber in the labyrinth. The proximal flaps lack these structures and therefore have a smoother outline. Between the distal flaps and the horizontal cavity, electron-lucent fibres are anchored in the wall of the horizontal cavity (Figure 2a). Between the two pairs of flaps, areas lacking any cell organelles are visible (Figure 2b). Two sheath cells enclose the support cell (Figures 3b and 3c). These cells extend furthest into the cuticular channel and surround the horizontal cavity and its connection to the labyrinth. All cell types (duct cell, support cell, sheath cells) entering the cuticular channel in the baseplate form strong extensions apically; these extensions are connected to each other by long septate desmosomes (Figures 3b and 3c).

The secretory cells contain granular secretion, often forming large deposits of different electron density within their lumen (Figure 2d). Fibrous structures may be present within the duct leading to the horizontal cavity (Figure 2b). Granular, electron-dense material is visible in the horizontal cavity. This material is often surrounded by an electron-lucent "halo" (Figure 2a). In the primary atrial chambers, no secretions are present.

Discussion

The large surface and position of the spiracles in *Ixodes ricinus* are typical for all Ixodidae, as opposed to Argasidae and *Nuttalliella namaqua* (Latif *et al.*, 2012; Sonenshine, 1991; Woolley, 1972). The gross morphology is in agreement with available descriptions of this and other ixodid species (Pugh *et al.*, 1988; Pugh, 1997): the connection between the labyrinth, the sub-ostial space and the tracheal atrium is clearly visible. Moreover, we confirmed connections between the chambers by fenestrations, the closed condition of the "ostium" (Hinton, 1967;

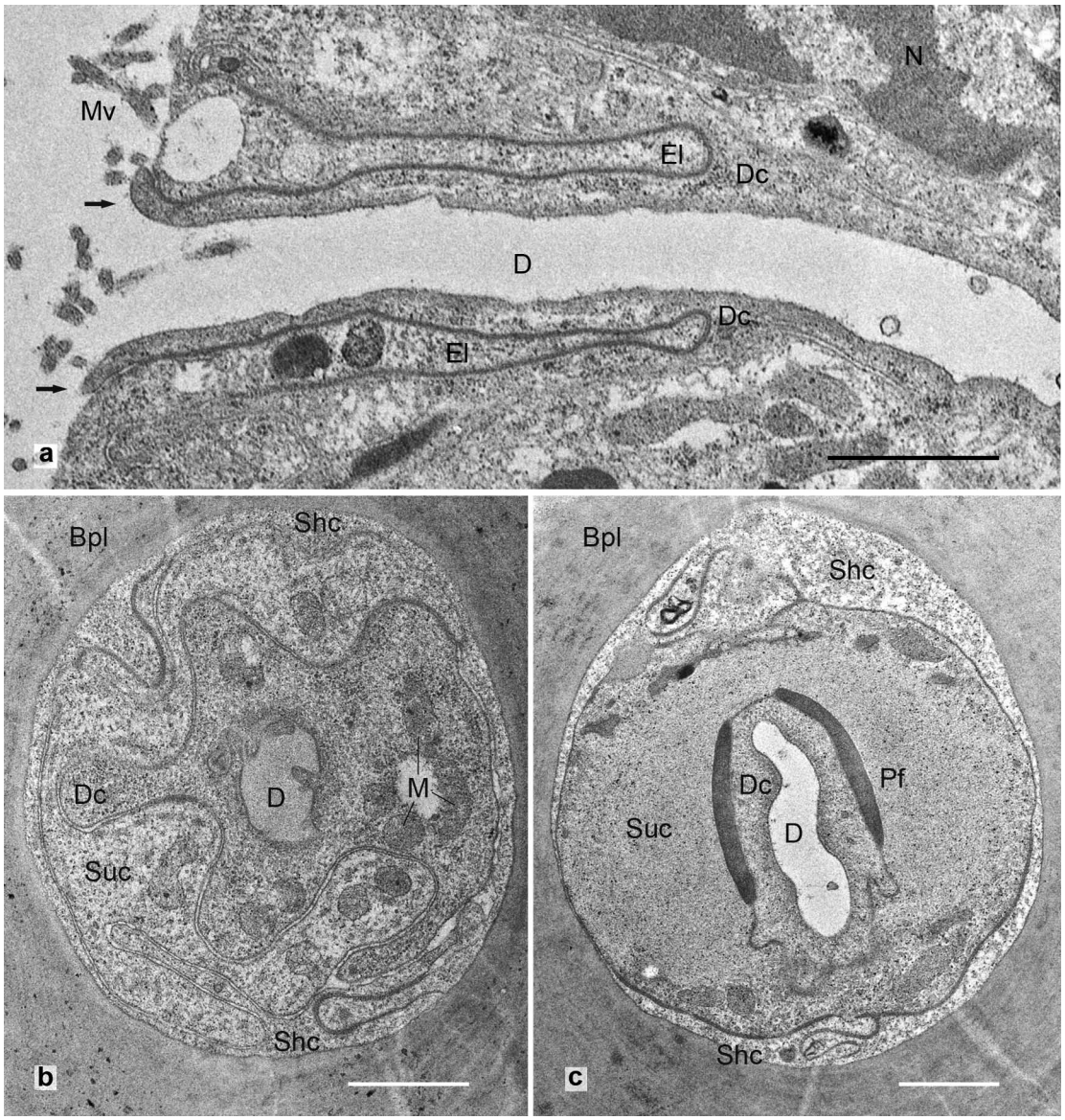


FIGURE 3: Transmission electron microscopy (TEM) images of spiracle gland cells in a male *I. ricinus*. a – Detail of the connection between the duct cell and the neck of a secretory cell. The duct cell sends narrow proportions along the inside of the neck slightly curving to the sides (arrows) where they reach the cup-shaped depression of the secretory cell. Note the dense membranes indicating cell junctions where the duct cell is attached to the neck of the secretory cell. Scale bar: 1 μm; b – Cross section in the lower part of a cuticular channel. On this level the gland cells show strong lateral protrusions linked to each other. A single duct is visible in the duct cell. Scale bar: 1 μm; c – Cross section of a cuticular channel on the level of the lower part of calyx region. The duct cell is surrounded completely by the support cell which is encircled by two sheath cells. Cell margins are irregular to a lesser extent and parts of the proximal flaps are visible. The dense membranes again indicate cell junctions. Scale bar: 1 μm. Bpl, baseplate; D, duct; Dc, duct cell; El, elongations of the secretory cell neck; M, mitochondria; Mv, microvilli; N, nucleus; Pf, proximal flap; Shc, sheath cells; Suc, support cell.

Pugh, 1997; Woolley, 1972) and the cuticular channels through the baseplate. The pedicels in *I. ricinus* have more than three edges, a condition typical for Ixodinae (Pugh, 1997).

The extension of the glands in the Ixodidae *I. ricinus*, *Haemaphysalis inermis* (Sixl and Sixl-Voigt, 1974) and *Rhipicephalus appendiculatus* (Walker *et al.*, 1996) is similar. In none of these species are the whole glands restricted to the region of the cuticular channels in the baseplate, as is the case in the "basal plate glands" of the ixodid *Amblyomma americanum* (Baker, 1997). In both *I. ricinus* and *A. americanum* (Baker, 1997), the cross section of the cuticular channel in the lower part shows lateral extensions of the gland cells. Cross sections through the glands of *I. ricinus* reveal that the duct is formed by a single cell only. This unusual nature of the duct is known neither from *Amblyomma americanum* (Baker, 1997), nor *Rhipicephalus appendiculatus* (Walker *et al.*, 1996) or *Haemaphysalis inermis* (Sixl and Sixl-Voigt, 1974). The dense and parallel arrangement of microtubuli in the duct cell and the support cell might prevent a collapse of the lumen. Lateral protrusions of the gland cells in the proximal region and long cell junctions clearly hold the cells in position. This is important when large amounts of secretions are transported or when the tick is feeding.

Since the gland cells are intact when there is little or no secretion in the cup-shaped cavities and the duct, a holocrine secretion as in other dermal glands of ticks (e.g., type 2 glands; Yoder *et al.*, 2009) can be ruled out. Instead, aggregations of numerous vesicles near the secretory cell membranes indicate merocrine secretion. The granular secretory product is similar in the vesicles, the duct and the horizontal cavity. The varying electron density of secretions indicates at least two components of the secretory products. The electron-lucent "halo" in the horizontal cavity could reflect the second component. Its peripheral position in the secretion may protect other components or facilitate secretion. Another possibility is a chemical reaction, probably by contact with air from the connection to the primary atrial chamber. The fibrous structures in the lumen of the duct leading to the horizontal cavity are probably aggregated granules of the secretory product.

An early light microscopic examination of the spiracle of a female *Hyalomma marginatum* describes secretory products within chambers of the labyrinth (Schulze, 1942). Some workers suggested that the secretion in the labyrinth closes the pores of the sieve plate, leading to the misinterpretation that there are no pores (Sixl and Sixl-Voigt, 1974); consequently, the "ostium" was interpreted as a functional opening for the gas exchange in Ixodidae. We detected no secretions within the chambers of *I. ricinus*. Secretion probably depends upon feeding by the ticks, as stated for *Haemaphysalis inermis* (Sixl and Sixl-Voigt, 1974). Alternatively, parts of the secretory product could be volatile.

If the destination of the secretion is the tick's environment, as would be the case for a pheromone, the labyrinth would provide a large surface for dispersion. Depending on the chemical composition of the secretion, the pores in the sieve-plate may allow the passage of only small portions of secretion. These pores may facilitate a constant discharge of the secretion over a longer period of time. Walking specimens of *I. ricinus* leave conspecific chemical cues of unknown origin on the substrate (Zemek *et al.*, 2002; Zemek *et al.*, 2007). Due to the posteriorly directed and substrate-facing position of the spiracles, their associated glands would be potential producers of these substances.

The cuticular apparatus of flaps under the horizontal cavity is termed calyx and is a typical feature of dermal glands in Anactinotrichida (Alberti and Coons, 1999). The calyx in *I. ricinus* resembles the structure described in *H. inermis* (Sixl and Sixl-Voigt, 1974). Two sclerites in an illustration of the spiracle gland in *R. appendiculatus* (Walker *et al.*, 1996) are presumably identical with the flaps (or only a pair of them) in *I. ricinus*. The electron-lucent fibres anchored in the horizontal cavity are probably part of an opening and closing mechanism of the calyx structure. The homogeneous areas lacking cell organelles between the two pairs of flaps indicate motility of these cuticular projections. Ultrastructural observations of dermal glands in other Anactinotrichida also show electron-lucent material in the calyx region, potentially the elastic protein resilin (Alberti and Seeman, 2005; Alberti, 2010).

TEM images from the calyx region in *A. americanum* indicate structural reduction. The L-shaped duct probably corresponds to one half of the horizontal cavity and its connection to the primary atrial chamber in the labyrinth. Even though Baker (1997) mentioned that the glands lack cuticular projections or flaps, an electron-dense structure underneath the L-shaped duct may be a small remnant of one flap. In the region of Haller's organ in the same species, a cuticular gland with four cuticular projections is described (Foelix and Axtell, 1972). This shows that the structural reduction does not affect all dermal glands in *A. americanum* but seems to be restricted to the region of the spiracle plate. The structural reduction does not necessarily functionally impair the glands, but the glands may be relict organs as suggested for type 1 glands in male *R. appendiculatus* (Walker *et al.*, 1996).

Following Pugh's idea of the hygroscopic function of the spiracle glands (Pugh, 1997), a reduction in *A. americanum* is plausible for several reasons: spiracles of Amblyomminae with small pore diameters and numerous densely packed pedicels are better suited for xeric environments. Moreover, reduced air flow simultaneously decreases water loss more effectively than in *I. ricinus* (Pugh, 1997). A SEM image of the spiracle plate from a female *A. americanum* (Baker, 1997) shows numerous, minute pores. Accordingly, a very dense array of pedicels must be present in the labyrinth of this species. Both the small pore diameters and the dense pedicel arrangement may be effective enough to prevent water loss, enabling a reduction of potential hygroscopic glands. *A. americanum* is known to imbibe liquid water (Benoit *et al.*, 2007). This is in contrast to *I. ricinus*, which depends on water vapour absorption (Kahl and Alidousti, 1997) by hygroscopic salt secretion of the salivary glands (Rudolph and Knülle, 1974).

Ticks are extremely successful: no other arthropod group is able to survive comparably long periods without food or drinking water. Nevertheless, desiccation is a major problem for ticks, especially in the off-host periods, which can last for more than 90 % of their life time (Needham and Teel, 1991). In addition to an impermeable cuticle (Fielden *et al.*, 2011), ticks have evolved behavioural and physiological adaptations to minimize water loss. Ticks aggregate in suitable microhabitats (Benoit *et al.*, 2007) and react to lengthier unfavourable conditions with diapauses (Sonenshine, 2005) and extremely low metabolic rates. The desert-inhabiting tick *Hyalomma dromedarii* searches for hosts in brief hunting phases instead of prolonged ambush. Between the attacks, individuals of this species are buried in sand, minimizing exposure to the dry environment (Randolph, 2004). Discontinuous ventilation also contributes to the water balance in ticks and is confirmed in members of Ixodinae (Slama, 1994) and Amblyomminae (Lighton *et al.*, 1993).

In *Ixodes ricinus* two functions of the spiracle glands must be considered. One is retaining water during respiration, as proposed by Pugh (Pugh, 1997), the other is providing intraspecific chemical cues. Based on our results the horizontal cavity is interpreted as a lock chamber separating the gland cells from the labyrinth and as a reservoir close to the final destination of the secretion. Further studies are required to investigate the role of the glands as potential sources for chemical cues or hygroscopic substances.

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