Morphology and ultrastructure of the epithelial femoral gland in cicadas (Hemiptera: Cicadidae)

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Abstract: Exocrine glands in the legs of social insects are found throughout all leg segments, but studies of exocrine glands in legs of solitary insects are very limited. We discovered a novel gland at the apex of the fore, mid and hind femurs from six representative species of Cicadidae, which we propose to name as the epithelial femoral gland. The epithelial femoral gland is located between the paired apodemes and the articulation membrane within the apex of the femur, which faces the proximal articulation region of tibia. The epithelial femoral gland in the midlegs is less developed than that in the fore- and hindlegs within a species. The glandular cells belong to class-1, which contain a large amount of rough endoplasmic reticulum, secretory vesicles and Golgi bodies, indicating these cells may produce a proteinaceous secretion. Details of the epithelial femoral gland at the ultrastructural level suggest that it may function to produce nourishing substances to the joint between femur and tibia. The less developed epithelial femoral gland in the midlegs and the slight difference in the glands between fore- and hindlegs within a species could be related to the functional differentiation of the corresponding legs in cicadas. Further studies of exocrine glands in the legs of cicadas and other Cicadomorpha insects may improve our understanding of the structural and functional divergence of legs in hemipteran insects.

Key words: legs, exocrine gland, ultrastructure, functional morphology, comparative morphology

1 Introduction

Insects, as the most species-rich of all animal groups on the earth, account for more than 50% of the biological world, and can be found almost everywhere in the world. The exocrine glands of insects are a response to the stimulation of the internal and external environment (Chen et al., 2003). The exocrine glands of insects are usually formed by specialized epithelial cells. Noirot and Quennedey (1974) divided gland cells into three classes according to the way secretions pass through the cuticle: class-1 gland cells are adjacent to the cuticle, and discharge their secretion directly through minute cuticular openings; class-2 gland cells were later identified as oenocyte homologues (Noirot & Quennedey, 1991); and class-3 gland cells are made up by bicellular units composed of duct cells and secretory cells, in which the secretions produced by secretory cells are carried to the exterior by a continuous cuticular ductule secreted by duct cells. Currently, most scholars accept that the exocrine glands of insects consist of class-1 and class-3 cells (Billen & Šobotník, 2015).

Exocrine glands are important organs of insects, which have been found all over the insect body (Hölldobler, 2016). The legs are the main locomotion organ of insects and also the predation tools of some insects. In social insects, Billen (2009) reviewed the occurrence and structural organization of exocrine glands in ant legs and found 20 different glandular structures, of which some can be involved in the secretion of trail pheromone chemicals used for communication. Hölldobler & Palmer (1989), Pouvreau (1991) and Hölldobler et al. (1992) described the exocrine glands present in the last tarsal segment and pretarsus of adult ants and bumble bees, which are responsible for the production of important compounds, including pheromones and antimicrobial peptides. Jarau et al. (2012) reported the structure of leg tendon glands in males of *Bombus terrestris*. Billen & Vander Plancken (2014) examined the organization of exocrine glands in the legs of the stingless bee *Frieseomelitta varia* and described 15 different glands. Nijs & Billen (2015) surveyed the structure of exocrine glands in the legs of the social wasp *Vespula vulgaris* and found 17 different

glands. Costa-Leonardo et al. (2015) investigated the exocrine glands in the tarsomeres and distal tibia in termites and described their structure and potential roles. Many exocrine glands have been found in the articulation region with the next leg segment, which can be involved in producing lubricant substances to reduce friction (Nijs & Billen, 2015; Billen & Vander Plancken, 2014).

However, our understanding of exocrine glands in the legs of solitary insects is very limited. Some papers have described glands in the tarsi and their involvement in the production of adhesive fluid in insect attachment to the surface (Bauchhenss & Renner, 1977 in flies; Kim et al., 2017 in bed bugs; Rebora et al., 2021 in bugs). Basitarsal glands have been described in the forelegs of male empidid flies, where they have a role during courtship (Young & Merritt, 2003), and in embyopteran webspinners which use their foreleg basitarsal glands to produce silk lining of the galleries under the bark of trees (Büsse et al., 2015). Some dung beetles have epithelial glands in their foreleg coxae and femurs that play a role in species recognition and sexual attraction (Houston, 1986).

Cicadas (Hemiptera: Cicadidae) are well known for their loud calling songs produced by the males (Young & Bennet-Clark, 1995). The family Cicadidae comprises approximately 3,100 species (Sanborn, 2013). Adult cicadas feed on xylem sap of host plants and live on the trunk of the canopy of trees, which require more leg strength and flexibility when compared to the nymphal cicadas. Given that the femur is the longest and most robust leg segment in cicadas, we regard the articulation region between femur and tibia as an example to explore whether exocrine glands exist in the articulation region of cicada legs. The anatomy and ultrastructure of the apex of fore, mid and hind femurs of six representative species belonging to five tribes of the subfamily Cicadinae in Cicadidae were observed using light microscopy (LM) and transmission electron microscopy (TEM).

2 Material and Methods

2.1 Insect species

Live adult males and females of six cicada species were collected by netting. The

species and collecting information are shown in Table 1. When specimens were collected and brought back to the laboratory, the apex of femurs were cut off from the legs and immediately fixed in cold 2% paraformaldehyde-2.5% glutaraldehyde for 12 h at 4 °C.

2.2 Light microscopy (LM) and transmission electron microscopy (TEM)

In total, eight specimens (four males and four females) for each species were observed using LM and TEM microscopy, respectively. For describing the position and details of the apex of femurs, photographs of the femurs of dry specimens were taken under a QImaging Retiga 4000R digital camera (CCD) (QImaging, Surrey, BC, Canada).

The fixed samples were rinsed with phosphate buffered saline (PBS, 0.1 M, pH 7.2) four times, and post-fixation was performed in 1% osmium tetroxide for 1.5 h, followed by the same rinse time and PBS. After dehydration in an ethanol series (30%, 50%, 70%, 80%, 90% and 100%) and 100% acetone, the tissues were infiltrated in a mixture of LR-White in acetone (1:3 for 2 h, 1:1 for 5 h, 3:1 for 12 h). Finally, samples were transferred to pure LR-White and embedded for 72 h. Tissues were sectioned with a Leica EM UC7 ultramicrotome (Hitachi, Tokyo, Japan). Semithin sections with a thickness of 1 μm were stained with toluidine blue and viewed in an Olympus BX-51 microscope (Olympus Corporation, Tokyo, Japan). Thin sections for electron microscopy with a thickness of 70 nm were double stained with lead citrate and uranyl acetate, and viewed in a Tecnai G2 Spirit Bio Twin electron microscope (FEI, Chech, USA).

2.3 Measurement and data statistics

We used Adobe Photoshop CS6 to measure the thickness of the epithelia at the apex of femurs, and then used Microsoft Excel 2010 for data statistics.

3 Results

The apex of femurs is slightly expanded laterally on each side of the femur in all

six sampled species (Fig. 1A), with an inwards extending apodeme underneath (Fig. 1B). The paired apodemes are adapted to provide attachment points for the muscles in the leg articulation region. A novel exocrine gland is found in six investigated cicada species between the paired apodemes and the articulation membrane at the apex of the femur, which forms the connection with the tibia (Fig. 1B). The apodemes vary in length within and among species. For example, the apodemes of the fore femur in *T. japonensis* are the longest (Fig. 2A), followed by that of the fore femur in *H. maculaticollis* and *K. caelatata* (Fig. 2C, E); the apodemes of the fore femur in *M. opalifera*, *M. mongolica* and *P. kaempferi* are relatively short (Fig. 2G, I).

Histological observations show that the epithelium lining the cuticle underneath the apex of femurs is thickened and specialized to form the gland in the legs of all six investigated species, which we propose to designate as the epithelial femoral gland (Fig. 2). The thickness of the glandular epithelium in the fore and hind femurs is obviously different from the normal tegumental epithelium elsewhere within a species. In contrast, the thickness of the glandular epithelium in the mid femur is slightly different from the normal tegumental epithelium (Table 2). The thickness of the glandular epithelium in the distal part of the hind femur is less than that of the fore femur within a species, e.g., in *T. japonensis* the glandular epithelium in the fore femur is 18.07 ± 2.97 μm (Fig. 2A) but only 10.53 ± 1.70 μm in the hind femur (Fig. 2B); in *P. kaempferi* the glandular epithelium in the fore femur is 27.09 ± 3.69 μm but only 8.94 ± 1.67 μm in the hind femur (Fig. 2L). The same phenomenon can be found in *H. maculaticollis* (46.65 ± 3.52 μm in the fore femur but 32.04 ± 4.57 μm in the hind femur) (Fig. 2C, D). In contrast, the epithelial femoral gland of the midlegs is less developed, e.g., in *H. maculaticollis* the glandular epithelium in the mid femur is only 19.71 ± 1.19 μm, which is much less than the thickness of the fore- and hindlegs (Fig. 2C, D, K). In addition, the thickness of the corresponding epithelial femoral glands is obviously different among species (Table 2).

Ultrastructural observations confirm that the glandular cells are obviously different from normal tegumental epithelial cells which have dilated intercellular spaces but no microvilli, and the large ovoid nuclei are located in the center of cells

(Fig. 3A, B). In contrast, the apical cell membrane of glandular cells of the epithelial femoral gland displays long and slender microvilli (Fig. 3C, D). They release their secretions directly through minute irregular pores (~0.2 μm diameter) to the exterior, i.e., the articulation region between the femur and the tibia (Fig. 3C, D). The large ovoid nuclei with evident heterochromatin bounded by nuclear membrane are located in the center of the glandular cells (Fig. 4A, B). Around the nuclei, scattered elements of a well-developed Golgi apparatus are found (Figs 3C, 4A). Septate junctions (Fig. 3C) and dilated intercellular spaces can also be observed in the glandular cells (Fig. 4C). The rough endoplasmic reticulum is typically arranged in parallel stacks and mainly located in the basal part of the cells and around the secretory vesicles (Fig. 4B). The basal plasma membrane of glandular cells is deeply invaginated into well-developed infoldings, which extend into the central region of the cells (Fig. 4C). Numerous mitochondria are associated with basal invaginations, and are scattered throughout the cytoplasm (Fig. 4C). The cytoplasm of the glandular cells contains abundant secretory vesicles with a diameter of ~0.5–1.0 μm (Fig. 4D). A large number of microtubules and bundles of microfilaments are distributed near the secretory vesicles and nucleus (Fig. 4D). A large number of secretory granules are scattered in the cytoplasm, with a diameter varying from ~0.5 to ~2.0 μm (Fig. 4D).

4 Discussion

Exocrine glands located at the apex of femurs were first found in social insects. Billen (2009) described the structural organization of the distal femoral gland in the mid and hind femur of workers of the ants *Diacamma vagans* and *Harpegnathos saltator*. Billen and Vander Plancken (2014) also reported the distal femoral gland in the fore and mid femur of the stingless bee *Frieseomelitta varia*. However, the function of the distal femoral gland may vary among different insects, e.g., it occurs in the femur of both males and females in polistine wasps and possibly plays a role in marking of flight paths (Beani & Calloni, 1991), whereas it may have a lubricant function in the heavily sclerotized species of ponerine ants (Billen, 2009).

In the present paper, we investigated the morphology and ultrastructure of the novel epithelial femoral gland in Cicadidae. The epithelial femoral gland is found in all legs of the six investigated cicada species, but the gland in the midlegs is less developed than that in the fore- and hindlegs. The gland is located between the paired apodemes and the articulation membrane which faces the proximal articulation region with the tibia. The apodemes, besides providing attachment points for the muscles in the femur, may provide protection and, possibly, also structural strength for the glandular cells, which are subjected to considerable mechanical forces due to their location in the articulation region between cuticular structures (Billen & Ito, 2006). The size of the apodemes varies within and among species (e.g., the apodemes in *M. mongolica* are smaller than those in *H. maculaticollis*), which may be related to the thoracic segment to which the leg is attached and to the body size of cicada species. Future comparative morphological studies of the apodemes in the fore-, mid- and hindlegs of cicadas within and among species may improve our understanding of the structure and function of these main locomotion organs in Cicadidae and other Cicadomorpha taxa such as Cicadellidae and Cercopidae.

The glandular cells of the epithelial femoral glands in cicadas join directly to the cuticle and lack associated duct cells, indicating they are glandular cells of class-1 (Noirot & Quennedey, 1974). Most exocrine glands that appear in the legs of insects, e.g., the exocrine glands in legs of bugs (Rebora et al., 2021) as well as the cicada gland here described, belong to class-1, with an exception of the basitarsal glands in Empididae and in Embioptera which are the only glands of class-3 (Young & Merritt, 2003). The epithelial cells have numerous basal invaginations and apical microvilli. These features are often found in epithelial glandular cells, such as the tibial gland of the ant *Crematogaster scutellaris* (Billen, 1984) and the distal tibial gland of termites (Costa-Leonardo et al., 2015). The presence of basal invaginations and apical microvilli provides an increased surface area, which is important for the uptake of precursor molecules from the haemolymph into the cytoplasm, and later for the release of the secretory products (Billen, 1991). The presence of dilated intercellular spaces provides also evidence of transport and uptake of materials, which was verified

for many exocrine glands (Costa-Leonardo & Haifig, 2010). In all investigated species, there are numerous vesicles in the glandular cells, which are common in arthropod epidermal glands (Jia & Liang, 2015; Costa-Leonardo et al., 2015). The vesicles in the glandular cells of the distal epithelia in the femur of the six observed cicada species, in addition to the large number of ribosomes and well-developed Golgi complexes, are probably directly involved in the synthesis and storage of the released substances. Šobotník et al. (2003) suggested that this type of cells have intense secretory activity, which can be powered by the large number of mitochondria in the cytoplasm.

In the present study, we observed that the secretory cells have well-developed rough endoplasmic reticulum and Golgi apparatus indicating the occurrence of proteinaceous secretions, which were also observed in the distal femoral gland of the social wasp *Vespula vulgaris* (Nijs & Billen, 2015). The abundant proteinaceous secretory granules observed in the distal femoral gland in cicadas suggest that these glandular cells may be mucus cells. The epithelial femoral gland in cicada species occurs at the inner side of the femur-tibia articulation, and therefore is well located to serve such nourishinig function. Details of the epithelial femoral gland at the ultrastructural level give further support for the suggested elaboration of proteinaceous exudates. The mucus cells secrete glycoproteins which combine with water to form mucus, which may provide nutritional substances (i.e., proteinaceous exudates) for the articulation membrane in the femur-tibia articulation.

The epithelial femoral gland in the midlegs is less developed than that in the fore- and hindlegs, and the thickness of the gland in the hind femurs is less than that in the fore femurs within a cicada species. This may be related to the different stress on the legs and the differences of legs in flexibility. In ants, the difference of flexibility may be responsible for the stress on different legs, i.e., the midlegs are less flexible than hindlegs, and the hindlegs are less flexible than the forelegs (Billen, 2008). As adult cicadas mainly perch on branches and trunks of trees with the head up, the stress on the forelegs and hindlegs is presumably greater than that on the hindlegs. The greater the stress on the legs, the greater structural strength between femur and

tibia, and the more nourishing substances are needed. The less developed epithelial femoral gland in the midlegs and also the slight difference in ultrastructure of the glands between the forelegs and the hindlegs could be closely related to the functional differentation of the corresponding legs in cicadas. Further investigations into the function of the epithelial femoral gland in cicadas are needed in future.

In conclusion, we hypothesize that the epithelia of the distal part of the femur in cicadas are specialized to form the epithelial femoral gland that secretes nourishing mucus (e.g., proteinaceous exudates). The epithelial femoral gland in the midlegs is less developed than that in the fore- and hindlegs, and the thickness of the glandular epithelia in the hind femurs is less than that in the fore femurs within a species. This could be related to the functional differentiation of different legs in cicadas. It is not clear whether cicadas have other exocrine glands in their legs. Further studies of exocrine glands in legs of cicadas and other Cicadomorpha insects are needed, which may improve our understanding of divergence of legs in structure and function in Auchenorrhynchan insects.

Author statement

LZ: conceptualization, species collection and identification, LM and TEM investigation, result interpretation, original draft, review & editing. SW: species collection and identification, review & editing. JB: result interpretation, review & editing. CW: conceptualization, result interpretation; original draft, review & editing, funding acquisition. All authors read and approved the final manuscript.

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Declaration of competing interest

The authors have no competing interests to declare.

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Table 1 Six representative species of the Cicadidae and their collecting information

Species	Tribe	Collection sites	Collection period
Platypleura kaempferi (Fabricius, 1794)	Platypleurini	Zhouzhi, Shaanxi Province, China	July 15, 2018
Meimuna opalifera (Walker, 1850)	Dundubiini	Zhouzhi, Shaanxi Province, China.	July 25, 2018
Tanna japonensis (Distant, 1892)	Leptopsaltriini	Ningshan, Shaanxi Province, China	July 31, 2019
Meimuna mongolica (Distant, 1881)	Dundubiini	Yangling, Shaanxi Province, China	August 3, 2018
Hyalessa maculaticollis (de Motschulsky, 1866)	Cicadini	Ningshan, Shaanxi Province, China	August 10, 2018
Karenia caelatata Distant,1890	Sinosenini	Ningshan, Shaanxi Province, China	August 15, 2018

Table 2 The thickness of the epithelium (μm) of six cicada species

Species	Legs	Range of thickness of glandular epithelium	Mean thickness of glandular epithelium (mean ± SE)	Mean thickness of normal epithelium (mean ± SE)
P. kaempferi	Forelegs	21.71 – 31.10	27.09 ± 3.69	10.43 ± 3.17
	Midlegs	7.31 – 8.53	7.92 ± 0.61	3.35 ± 1.23
	Hindlegs	7.30 – 10.77	8.94 ± 1.67	3.04 ± 1.13
M. opalifera	Forelegs	15.10 – 19.71	17.51 ± 1.38	4.79 ± 0.93
	Midlegs	10.09 – 13.75	11.39 ± 1.73	6.29 ± 1.92
	Hindlegs	14.42 – 17.98	15.56 ± 1.09	5.26 ± 0.56
T. japonensis	Forelegs	15.10 – 23.46	18.07 ± 2.97	5.70 ± 0.91
	Midlegs	6.15 – 8.55	7.83 ± 1.16	2.48 ± 0.86
	Hindlegs	8.75 – 13.02	10.53 ± 1.70	3.73 ± 0.69
M. mongolica	Forelegs	17.07 – 21.95	19.66 ± 2.01	8.73 ± 0.70
	Midlegs	10.09 – 11.63	10.60 ± 0.88	6.29 ± 1.92
	Hindlegs	10.19 – 15.19	12.49 ± 1.80	7.50 ± 0.34
H. maculaticollis	Forelegs	44.16 – 49.13	46.65 ± 3.52	9.23 ± 2.08
	Midlegs	18.36 – 21.06	19.71 ± 1.19	5.86 ± 1.08
	Hindlegs	29.88 –38.83	32.04 ± 4.57	8.53 ± 3.50
K. caelatata	Forelegs	25.19 – 35.19	28.36 ± 3.61	8.01 ± 2.04
	Midlegs	11.35 – 12.69	11.89 ± 0.73	9.26 ± 0.73
	Hindlegs	11.68 – 20.67	14.65 ± 3.15	9.10 ± 0.77

Figure legends

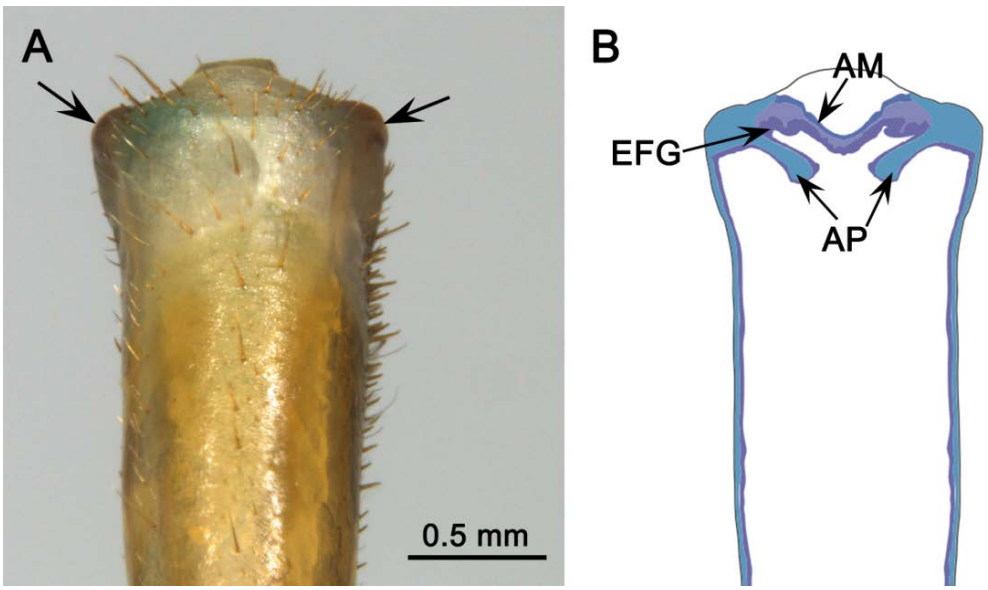
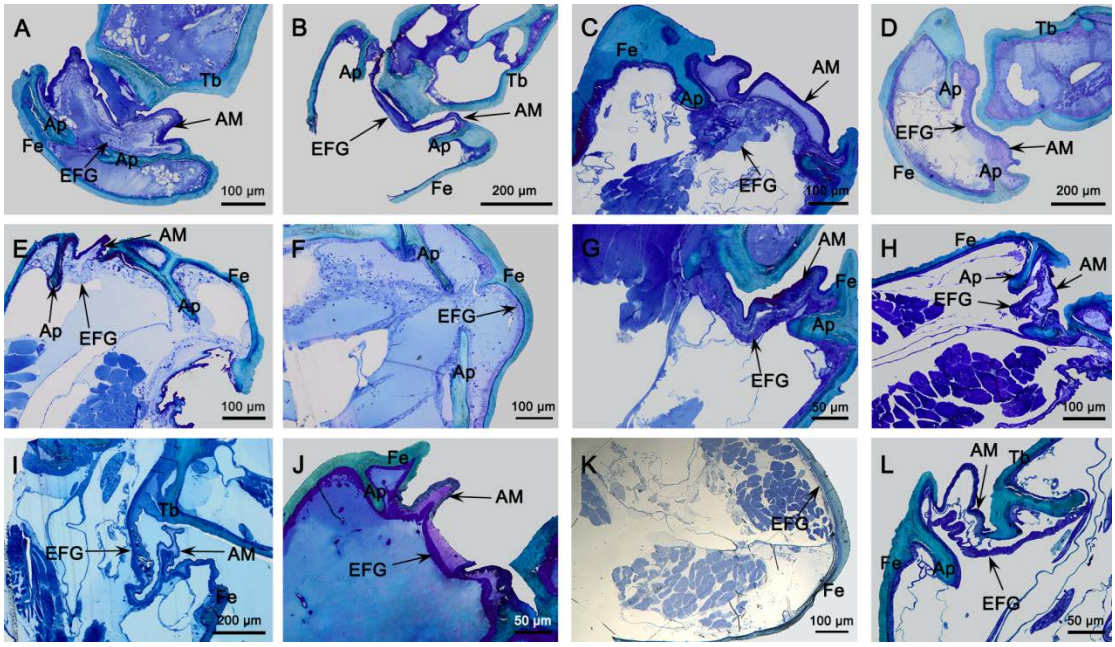


Fig. 1. Gross morphology of the distal part femurs. Arrows indicate the area where the cuticle extending inwards to form the apodeme. **A.** Dorsal view of the distal part of left hind femur of *M. mongolica*. **B.** Schematic representation showing the apodeme and epithelial femoral gland in the distal part of femurs. Ap = apodeme, AM = articulation membrane, EFG = epithelial femoral gland.



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336 **Fig. 2.** Longitudinal semithin histological sections through the apex of femurs. **A.** *T.*
337 *japonensis* (foreleg). **B.** *T. japonensis* (hindleg). **C.** *H. maculaticollis* (foreleg). **D.** *H.*
338 *maculaticollis* (hindleg). **E.** *K. caelatata* (foreleg). **F.** *K. caelatata* (midleg). **G.** *M.*
339 *opalifera* (foreleg). **H.** *M. opalifera* (hindleg). **I.** *M. mongolica* (foreleg). **J.** *M.*
340 *mongolica* (hindleg). **K.** *H. maculaticollis* (midleg). **L.** *P. kaempferi* (hindleg) Fe =
341 femur, Tb = tibia, Ap = apodeme, AM = articulation membrane, EFG = epithelial
342 femoral gland.

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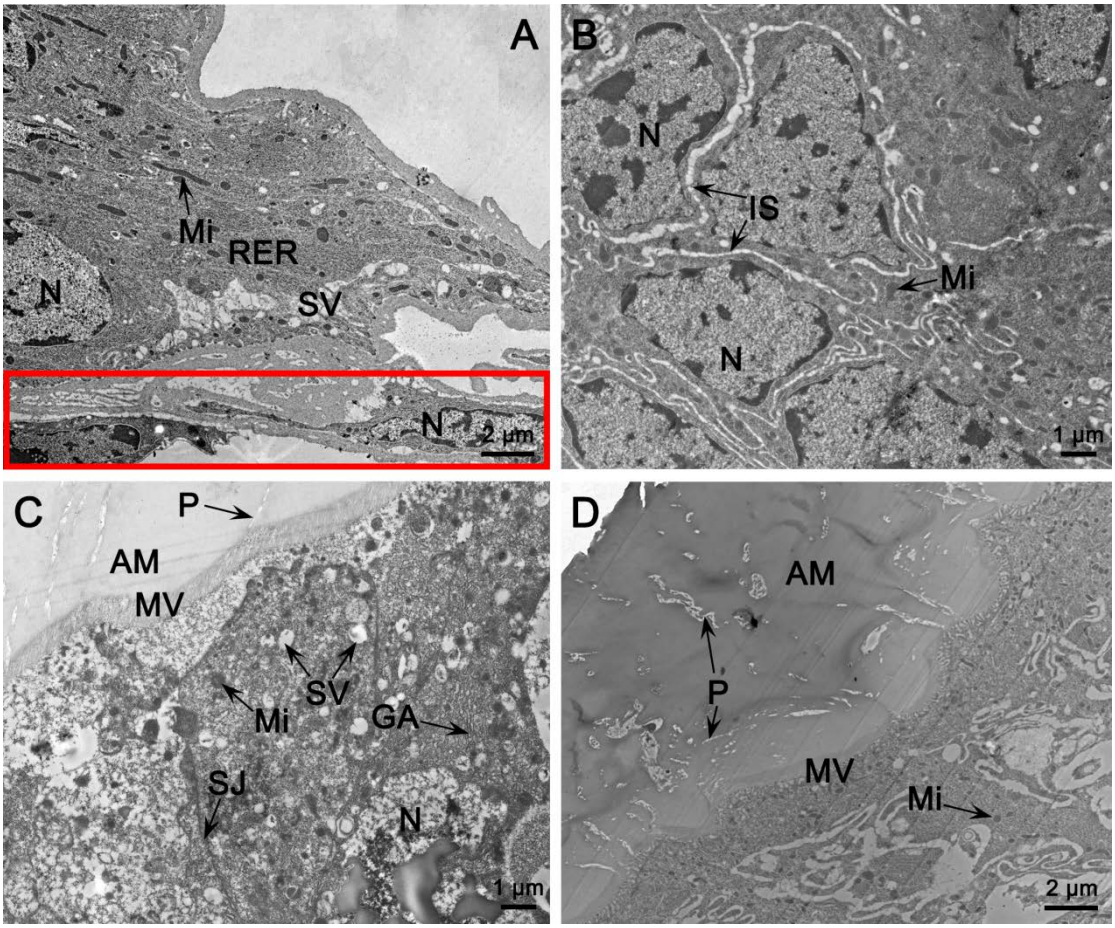
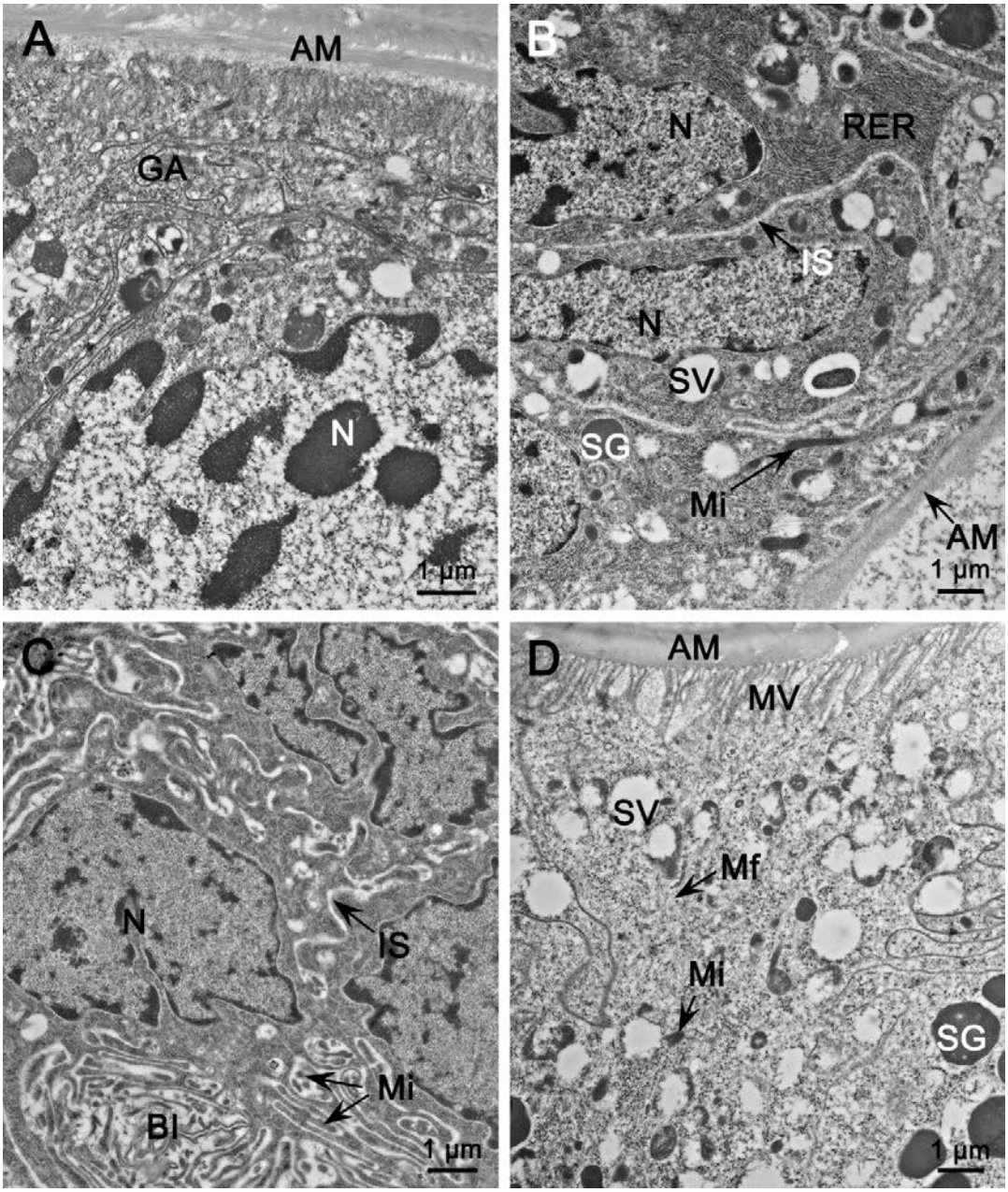


345 **Fig. 3.** Electron micrographs of longitudinal sections of the epithelial femoral gland.
346 **A.** Gland epithelium and non-glandular flat epithelium (in the red box) in hind femur
347 of *M. mongolica*. **B.** Non-glandular flat epithelium in hind femur of *M. mongolica*. **C.**
348 Apical part of the gland epithelium in fore femur of *K. caelatata* showing apical
349 microvilli and irregular pores. Inset shows irregular porese. **D.** Epithelial femoral
350 gland in fore femur of *M. opalifera*, showing pores in articulation membrane. AM =
351 articulation membrane, GA = Golgi apparatus, Mi = mitochondria, Mv = microvilli, N
352 = nucleus, P = pore, RER= rough endoplasmic reticulum, SV = secretory vesicle, SJ =
353 septate junction, IS = intercellular spaces.
354



355 **Fig. 4.** Electron micrographs of longitudinal sections of the epithelial femoral gland.
356 **A.** Glandular cells in the fore femur of *T. japonensis* showing well-developed Golgi
357 apparatus. **B.** Glandular cells in hind femur of *H. maculaticollis* showing numerous
358 secretory vesicles and secretory granules. **C.** Numerous basal invaginations of
359 glandular cells in hind femur of *M. mongolica*. **D.** Gland epithelium in hind femur of
360 *P. kaempferi* showing numerous microfilaments. AM = articulation membrane, BI =
361 basal invaginations, GA = Golgi apparatus, Mf = microfilaments, Mi = mitochondria,
362 Mv = microvilli, N = nucleus, RER= rough endoplasmic reticulum, SG = secretory
363 granule, SV = secretory vesicle, IS = intercellular spaces.