Wax glands of the horned gall aphid, *Schlechtendalia chinensis*, at different stages

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Abstract: The horned gall aphid, *Schlechtendalia chinensis*, inhabits the productive species of Chinese gallnuts, which have economic value. Aphid wax glands are crucial for the survival of the insects since the secreted waterproofing wax is important to protect the aphids from predators, pathogens and honeydew contamination. In this study, we investigated the structure of wax glands and their role in different aphid stages using light and electron microscopy. Our results showed that aphids of all stages except the newly hatched fundatrix possess six parallel dorsal lines with wax gland plates, including two dorsal, two dorsolateral and two lateral lines. Each aphid has a total of 56 wax gland plates with 11 dorsal, 9 dorsolateral and 8 lateral plates. Although no wax glands occur on the dorsum of the newly hatched fundatrix (first instar), the glands do appear once a fundatrix entered the second instar. The wax gland plate is composed of 2 to 22 polygonal depressions, each of which corresponds to a secretory cell covered by cuticle. The wax glands of this aphid belonged to the class 1 glands, which are formed by epidermal secretory cells. The structure of the wax glands varies in the different stages and these changes may be adaptive to the changeable microenvironments in which the aphids live.

Key words: *Schlechtendalia chinensis*; horned gall aphid; wax gland; morphology; ultrastructure; ecological adaptation

1. Introduction

Chinese gallnuts on *Rhus* trees (Anacardiaceae) display an abnormal growth that is induced by aphid feeding (Blackman and Eastop, 2000). The galls are valuable for both industrial and medicinal purposes due to their high level of tannin acids. There currently are 10 species and 4 subspecies of galling aphids

(Hemiptera: Aphididae: Eriosomatinae) in China. Among them, *Schlechtendalia chinensis* induces horned-galls on the Chinese sumac, *Rhus chinensis*. *S. chinensis* is a highly productive species (Zhang and Zhong, 1983). The life cycle of *S. chinensis* includes sexual as well as asexual reproduction stages with a host switch between *R. chinensis* and certain mosses (Mniaceae), such as *Plagiomnium maximoviczii* (Yang *et al*., 2010). At the beginning of spring, aphid nymphs develop into alate spring migrants (sexuparae) in mosses. Once the sexuparae migrate to nearby host trees, they produce male and female offspring (sexuales) in the trunk crevices. After mating, a female lives about a month and produces a fundatrix, which crawls along the trunk and feeds on a new leave, where it initiates gall formation. The fundatrix feeds continuously inside the gall and produces fundatrigeniae via thelytokous parthenogenesis. The gall continues to grow under the stimulation of feeding by the fundatrix and her fundatrigeniae offspring. In autumn, after galls mature and burst open, alate autumn migrants fly through the openings to nearby mosses for overwintering. In the next spring, nymphs develop into spring migrants and begin a new cycle (Zhang and Zhong, 1983). The complex life cycle with various morphological aphid types is mainly driven by adaptation to environmental changes (Moran, 1989; Liu *et al*., 2014).

Aphids feed on plant sap, which is rich in sugars but poor in lipids, which raises the question why they invest a lot of energy to produce lipids to cover their bodies. The roles of waxes secreted by three aphid species, *Phyllaphis fagi*, *Eriosoma lanigerum* and *Pachypappa vesicalis* are thought to prevent aphids from honeydew contamination, fungal infection, attack from parasites and predators, and chilling frost (Smith, 1999). In gall-living species, a wax coating protects them from being immersed in their own honeydew and reduces the risk of fungal infection. In free-living species, wax protects them against both natural enemies and adverse environmental conditions (Pike *et al.*, 2002; Moss *et al.*, 2006). In some Hemipterans, waxes play a special ecological role for environmental adaptation. For example, the males of a scale insect, *Ericerus pela* live under a wax cover, which may provide shade from strong direct sunlight, and allows the penetration of scattering light for their development needs to adapt to environmental changes (Qi *et al.*, 2019).

The morphology of the wax glands, wax gland openings and waxy secretions has been studied in several aphids, mealybugs and other Hemipterans, which showed variable structures among species (Pope, 1983; Smith, 1999; Lucchi and Mazzon, 2004; Ammar *et al.*, 2013; Pikart *et al.*, 2014). The number and distribution of wax glands in Hemipterans can either be two, four, or six rows of glands on the dorsal body surface or glands irregularly scattered on the back of the insect. The six rows of dorsal wax gland plates usually consisted of two rows of dorsal plates, two rows of dorsolateral plates and two rows of lateral plates (Chen and Qiao, 2012). The structural and quantitative changes of wax gland plates in the same species are related to the various activities associated with different life stages. The wax gland plates of aphids in Hormaphidinae (Hemiptera: Aphididae) are highly diversified in distribution, degree of development, shapes and structures as a result of adaptation to specific environments. Wax gland plates were shown in nymphs of several Hormaphidinae aphids but disappeared or are replaced by wrinkles in adults (Chen and Qiao, 2012). The gland amounts and types in early instar nymphs of *Carsidara limbata* are less than those in late instars (Li *et al.*, 2018). These ontogenetic changes appear related to different activity levels of each life stage (Chen and Qiao, 2012).

The horned gall aphid *Schlechtendalia chinensis* has a typical life cycle with six stages living in different microenvironments. This is especially the case for the fundatrix and autumn migrants which are living in both closed (inside a gall) and open environments (out of a gall) (Shao *et al.*, 2012; Wang *et al.*, 2020). Previous studies have focused on the aphid's life cycle, biological characteristics and interaction with its host plants, but only a few on its wax glands and functions. In this paper, the structure, quantity and distribution of wax gland plates of the horned aphid in all stages of its life cycle were investigated by light as well as scanning and transmission electron microscopy, to further explore their function and ecological significance. Understanding these wax gland changes in different stages may help to elucidate how the aphids adapt to the changeable microenvironments in which they live. Our purpose is to provide a theoretical basis for the study of ecological adaptability of wax glands and a scientific basis for the improvement of artificial breeding technology of the horned gall aphid.

2. Materials and methods

2.1. Insect sample collection

Aphids were collected from mature galls on the host tree, *Rhus chinensis*, growing in the field of Yanjin county (28°06′ N，104°22′ E，980 m above sea level), Yunnan Province. The samples of each aphid stage were obtained from artificially cultivated galls. Specifically, alate migrants were collected from mature galls in the fall and transferred to a nursery of the moss, *Plagiomnium maximoviczii* in a greenhouse. The following year, aphids were collected from the mosses and transferred to host trees for gall formation. Subsequently, aphids at different stages were collected from galls on host trees. The aphid stages used in this study are overwintering nymphs in mosses, spring migrants, sexuales including males and females, fundatrix, fundatrigeniae in galls, and autumn migrants.

2.2. Light microscopy

Aphid samples were soaked in cold 2% glutaraldehyde, then transferred to Na-cacodylate buffer (pH 7.3) for 12 h, and fixed in 2% osmium tetroxide. After dehydration in a graded acetone series, aphids were embedded into araldite and sectioned using a Leica EM UC6 microtome. Serial semithin sections with a thickness of 1 μm were stained with methylene blue and thionin, and observed under an Olympus BX-51 microscope.

2.3. Transmission electron microscopy (TEM)

To allow sufficient penetration of the various chemicals used during tissue processing, aphids were transversely cut to separate the anterior and posterior part. These body parts were fixed in cold 2.5% glutaraldehyde in Na-phosphate buffer (100 mM, pH 7.2) for 12 h and postfixed in cold 1% osmium tetroxide for 12 h. After dehydration in a graded acetone series, they were embedded in Araldite and sectioned using a Leica EM UC6 microtome. Thin sections with a thickness of 70 nm were double-stained with lead citrate and uranyl acetate and examined under a Zeiss EM900 electron microscope.

2.4. Scanning electron microscopy (SEM)

Aphid samples were cleaned and dehydrated with a graded ethanol series (70%, 80%, 90%, 95%, and 100%) and then were placed onto aluminium stubs using double-adhesive tape and then coated with gold in a HTC JS-1600 ion coater for 90 s and observed under a low-vacuum tabletop electron microscope Hitachi TM3000. Other samples were observed under the same microscope directly without any prior preparation or coating in order to see the wax filaments under natural condition. In each sample, 15-20 aphid individuals of each stage were examined.

3. Results

3.1. Distribution of wax glands in different stages

Wax glands occur on the dorsum of all aphid stages except for the newly hatched fundatrix. All observed aphids have a similar arrangement of dorsal wax glands at different stages. The wax gland plates are arranged in six parallel longitudinal lines on the dorsum, with two dorsal, two dorsolateral, and two lateral lines (Fig. 1A, B). For the dorsal lines, two wax gland plates are located on the head dorsum, one on the thorax notum and eight on the abdominal tergites. For the dorsolateral lines, one gland plate is on the thorax notum and eight on the abdominal tergites. For the lateral lines, eight gland plates are on the abdominal tergites while none occurs on the head dorsum or thorax notum (Fig. 1A-H). Each aphid therefore has a total of 56 wax gland plates comprising eleven on the dorsal lines, nine on the dorsolateral lines and eight on the lateral lines (Fig. 1A).

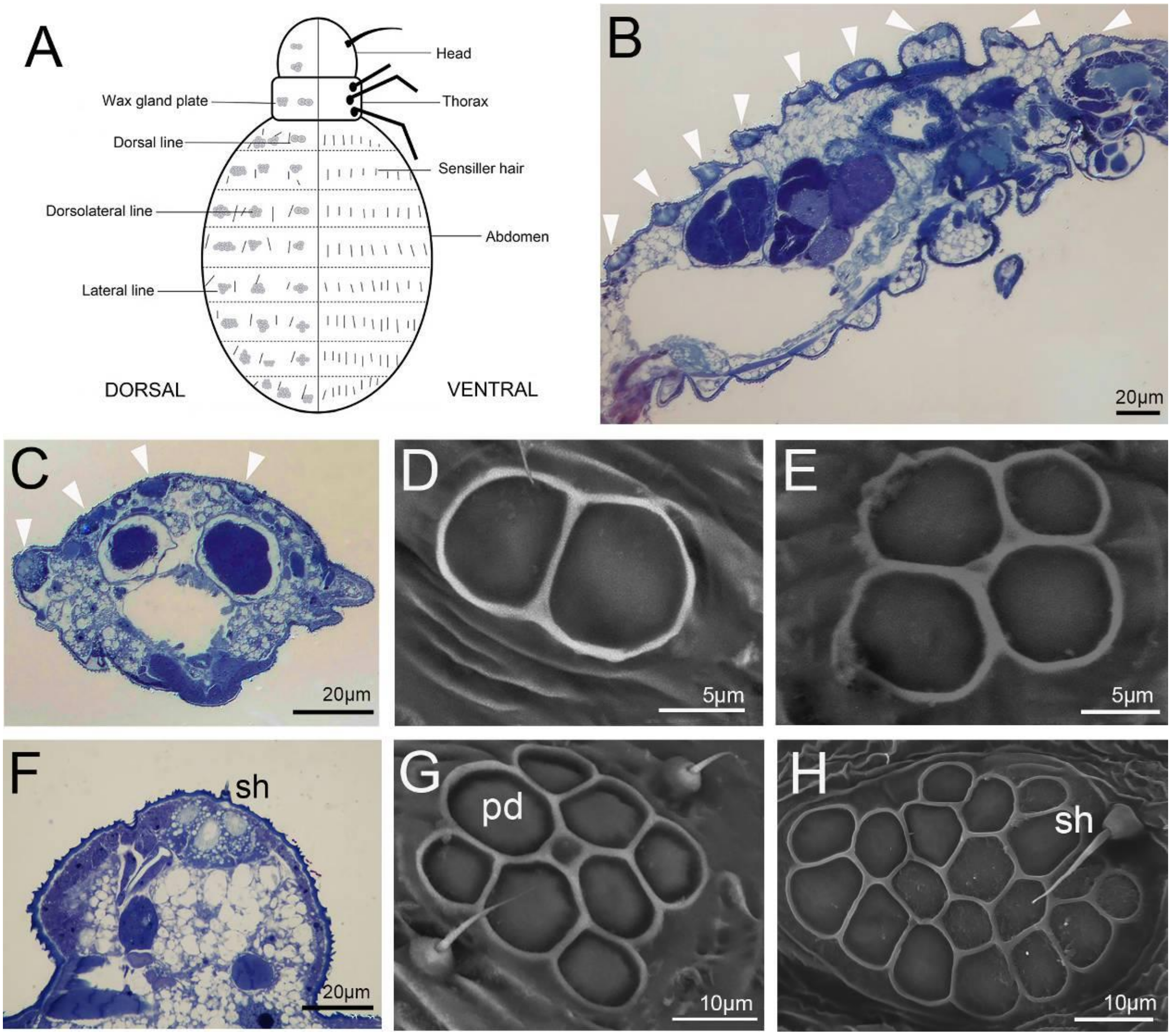

Fig. 1. Light and scanning micrographs of wax glands of the horned gall aphid. (A) Six rows of wax glands symmetrically distribute on the dorsal side of head, thorax and abdomen. (**B**) Semithin longitudinal section of galling aphid nymphs showing a line of wax glands on the dorsum. (**C**) Semithin section of galling aphid nymphs which shows wax glands on the dorsum. **(F**) Single wax gland. **(D), (E), (G), (H)** Wax gland plates with varying numbers of polygonal depressions. **Abbreviations:** arrowheads **-** wax gland plates; pd **-** polygonal depression; sh **-** sensillar hair.

Each wax gland plate is composed of various polygonal depressions with a diameter between 5 and 10 μm and are generally associated with one or two sensillar hairs with a length around 10 μm (Fig. 1G, H).

The polygonal depression numbers on each wax gland plate reflect its complexity. Generally, the more polygonal depression numbers, the more elaborate the structure. The number of polygonal depressions in each wax gland plate varies in different rows and on different dorsum parts. There are more polygonal depressions on the lateral lines than on the dorsolateral and dorsal lines. The number of the polygonal depressions on each wax gland on the head dorsum usually is only 2-5, with a relatively simple structure (Fig 1D, E). On the thorax notum, each wax gland plate containss 6-10 depressions with more complex structures (Fig. 1G). On the abdominal tergites, each wax gland plate containss 11-22 polygonal depressions, representing the most complex structures among the three body parts (Fig. 1H).

The number of polygonal depressions of each wax gland plate also varies among the aphid stages or forms. The wax gland plates of nymphs, spring migrants and autumn migrants are more complex than those in the other stages. The number of polygonal depressions in nymphs or spring migrants could reach more than ten, with each wax gland plate associated with one or two sensillar hairs. Also in fundatrigeniae, about ten polygonal depressions were observed in each wax gland. The number of polygonal depressions in sexuales is six to eight and is less than in aphids from other stages. The most striking observation is that there are no wax glands on the entire body of the newly hatched fundatrix (first instar). However, wax glands appear on the lateral lines of bodies after the first molting (second instar) (Fig. 2A-D). This finding was also confirmed in semithin sections of the fundatrix (Fig. 2C, D). Along with the occurrence of wax glands, the shape and color of their bodies change as well. The newly hatched fundatrix (first instar) lives outside the gall and their black bodies are slender without wax glands (Fig. 2A, B). After this stage feed on the tender leaves, atypical development starts on the leaves resulting in the formation of a gall with the aphid wrapped inside. The body of the aphid turns light yellow and becomes short and thick after the first molting (second instar) (Fig. 2C, D).

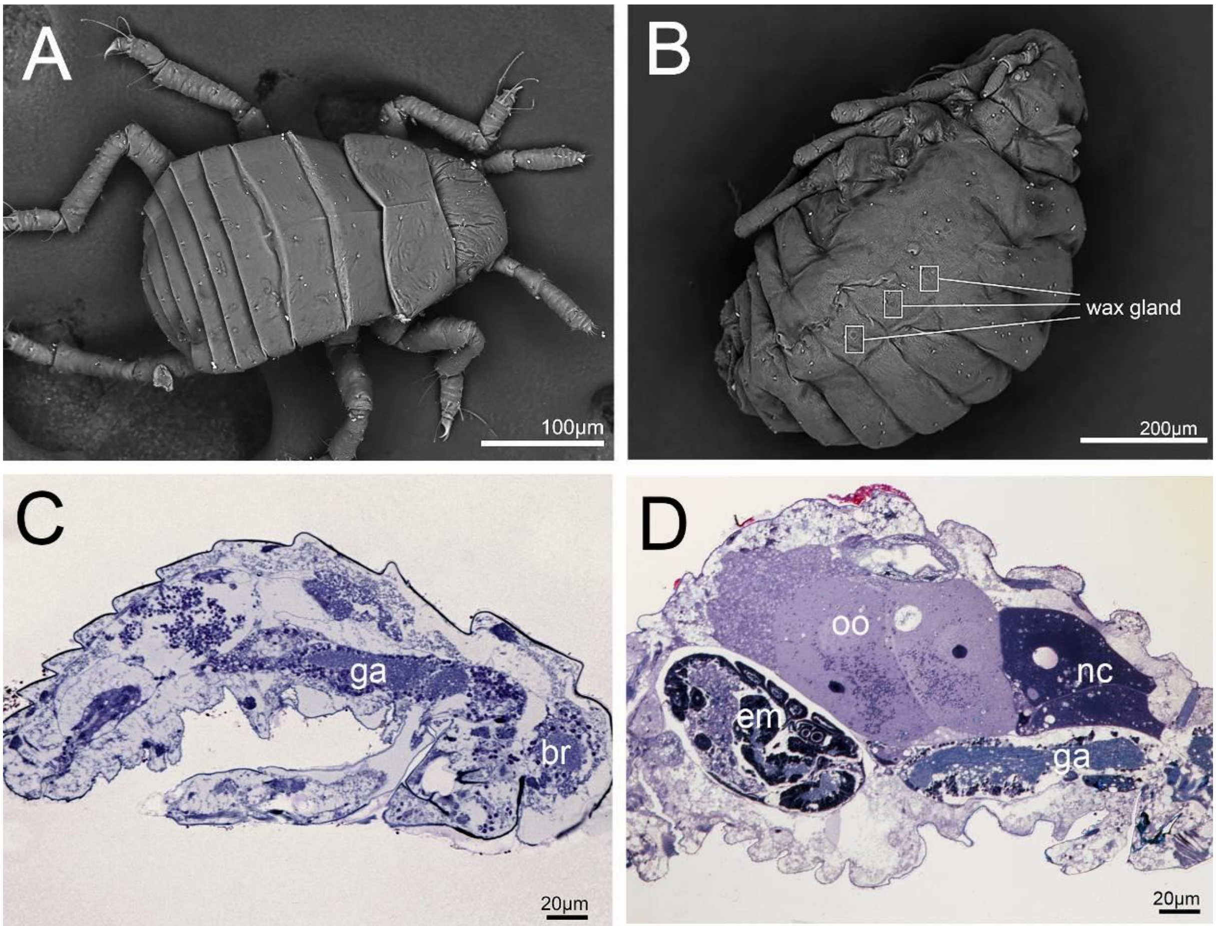

Fig. 2. Newly hatched fundatrix (first instar) and the fundatrix after first molting (second instar). (A) Newly hatched fundatrix (first instar); (**B)** First molting fundatrix (second instar); (**C), (D)** Light microscopy with longitudinal sections of first instar fundatrix (C) and abdomen of second instar fundatrix (D). **Abbreviation:** br - brain; em - embryo; ga - ganglion; nc - nurse cells; oo - oocyte.

3.2. Structural and functional analyses of wax glands at different stages

Each wax gland plate is composed of many polygonal depressions, which are arranged like a 'rosette' (Fig. 1G, H). Ultrastructural observation revealed that each polygonal depression corresponds to a secretory cell (Fig. 3B). The secretory cells have a height around 15 μm, and are directly covered by the tegumental cuticle, their apical cell membrane being differentiated into long and slender microvilli of up to 5 μm (Fig. 3A-C). A large subcuticular space of 5-10 μm locates between the microvilli and the cuticle acts as a kind of reservoir space, and there are a lot of electron-lucid vesicles in the cells (Fig. 3A). The gland cells contain many mitochondria and an abundant smooth endoplasmic reticulum (SER), of which strands penetrating into the apical microvilli could be seen (Fig. 3D-E). Locally, circular accumulations of rough endoplasmic reticulum in wax gland cells can be observed (Fig. 3F). The cells are arranged in a regular way without folding or overlapping of cell membranes. Adjacent cells are connected in the same way as other secretory cells, with a septate junction and more basally free space between the cells (Fig. 3D). The structure of cuticle can be divided into an electron-dense epicuticle of 0.1 μm, a fibrillar exocuticle of 1 μm, and an electron-lucid endocuticle of variable thickness. The exocuticle overlaying the wax gland plates appears darker than in the non-glandular region (Fig. 3G, H). The thickness of the endocuticle in the gland region is up to 10 μm, and is modified into a subcuticular space covering the glandular epidermis, whereas in the non-glandular region it measured around 1 μm.

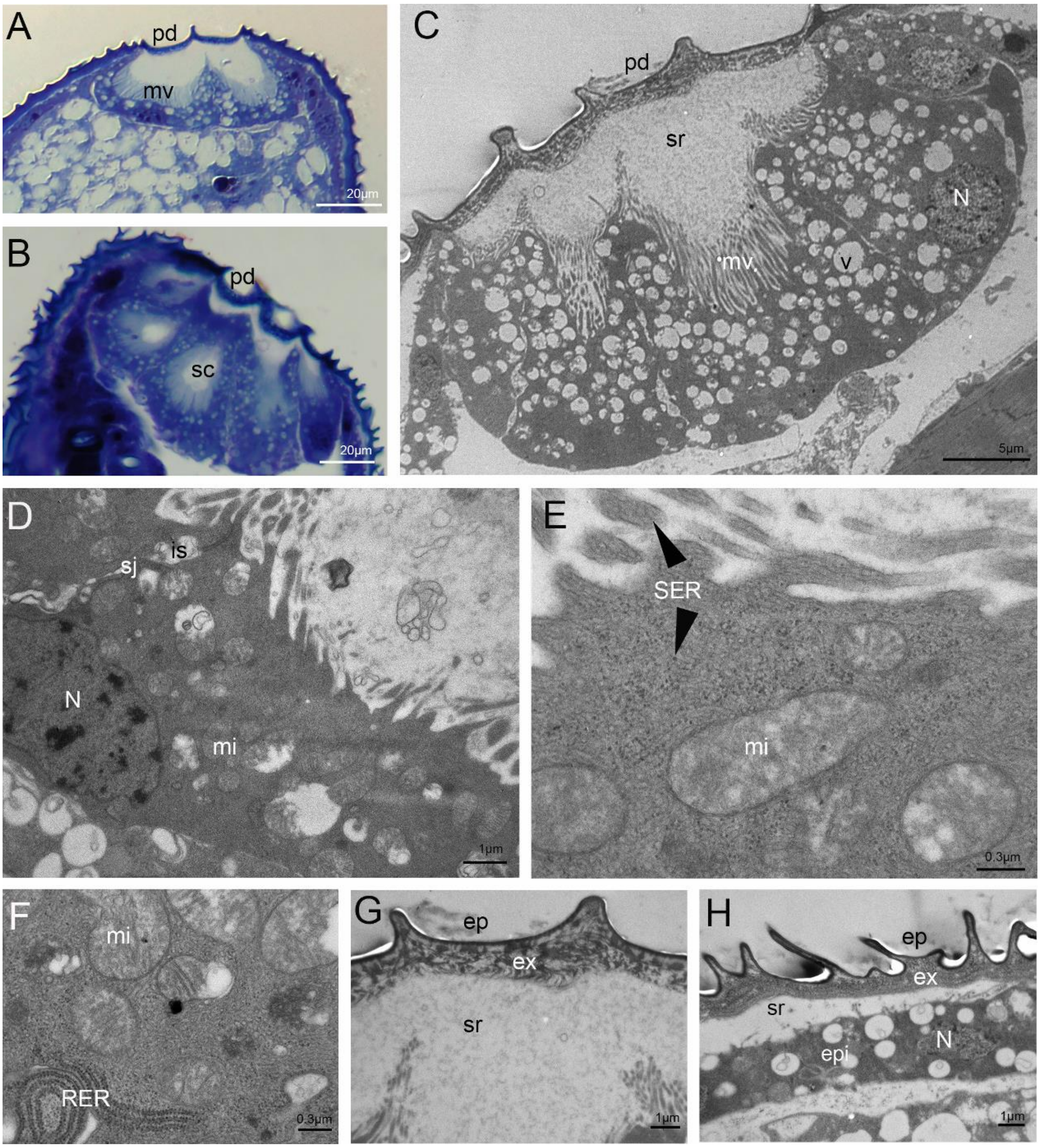

Fig. 3. Light microscope and TEM micrographs of wax glands. (A), (B) Semithin sections of wax glands. (**C)** Electron micrograph of wax glands. (**D**) Intercellular junction with a septate junction (sj). (**E**) Part of wax gland cell of *Schlechtendalia chinensis* showing the apical microvilli, with clearly visible strands of smooth endoplasmic reticulum (SER) inside them. (**F**) Higher magnification of wax gland showing mitochondria (mi) SER. (**G**) Cuticle covering glandular cells. (**H)** Cuticle covering non-glandular epidermis. **Abbreviation:** ep - epicuticle; epi - epidermis; ex - exocuticle; is - intercellular space; mv - microvilli; N - nucleus; pd - polygonal depression; RER - Rough endoplasmic reticulum; sc - secretory cell; sr - subcuticular space; V - secretion vesicles.

4. Discussion

4.1. Function and structure of wax glands in each stage are closely related to living environment of the aphids

Wax-secreting glands are special structures formed during the long-term evolution of insects (Pope, 1983). The structural complexity of wax gland plates varies in different rows and on different dorsum parts. The number of polygonal depressions per wax gland plate on the lateral row is higher than on the dorsolateral and dorsal rows. This may be an adaptation of aphids to the environment, since horned gall aphids often contact the wet surface of host plants by their lateral sides, where higher wax cover on their bodies may be helpful to protect them against excessive water. Similarity, the number of polygonal depressions on the abdominal tergites is higher than on the head dorsum and thorax notum. This may be because the abdominal tergites account for the largest proportion of the whole body (Fig. 1A) and moreover, its surface is softer than the head dorsum and thorax notum, which makes the need for wax protection more essential.

The functions and structures of wax glands in each stage of *S. chinensis* are also closely related to the environment in which they live. The overwintering nymph displays the most complex wax glands among all stages which secretes a lot of wax to form a wax ‘coating’ to cover itself and live underneath it for about four months, from November to next March. This wax ‘coating’ may help to keep water away from the body since the moisture around the moss layers is very high, and its relative humidity is nearly 100% (Wang *et al.*, 2020). Moreover, it may help the nymph to resist the drastic change in temperature and the attack from predators and pathogens. The wax ‘coating’ is almost the only protection for a nymph since it often feed on a moss twig stationary and it almost immobile. Previous studies showed that aphid wax covering had an anti-predator function (Moss *et al.*, 2006). Spring and autumn migrants have developed wax glands which may secrete wax for waterproofing and reducing heat desiccation. In early spring, the nymphs develop into winged migrants in mosses and will migrate to *Rhus* trees no matter the weather conditions. Because the weather is often changing quickly in this season, which is usually cold and rainy, and sometimes even alternating between freezing and thawing, the migrant aphids are forced to hide in moss layers waiting for suitable weather (Zhang and Zhong, 1983). The continuous rainfall and excessive moisture in moss layers are critical for the survival of migrant aphids. The complex wax glands of spring migrants may secrete lots of wax to cover their bodies as an anti-wetting coating which will protect them against raindrops and keeping the wings dry. Aphids fly from mosses to their host trees at once when the rainfall stops. Previous studies have shown that the wax coat with a bloom of wax filaments would help aphids to reduce the rate of heat dissipation by its hydrophobicity and the air that is trapped between the wax threads (Smith, 1999). A wax coating of autumn migrants would protect them from water and reducing heat desiccation as well. The winged autumn migrants of *S. chinensis* migrate from the crevices of dehiscing mature galls to nearby mosses in mid-autumn. The weather during this period is getting cold and sometimes is even frosty. Their bloom wax coats would help them to avoid excessive moisture in moss layers and adapt to lower temperature outside the closed galls. Sexuales of *S. chinensis* including males and females have more simple wax glands than overwintering nymphs, and migrants. They often live concealed in cracks or crevices on the surface of host tree trunks after mating. Since the humidity in the cracks or crevices is relatively high, a certain amount of wax on their body surface may help to prevent dew and also allows them to move for a short distance.

It is interesting that the newly hatched fundatrix is the only stage without wax glands. However, wax glands soon develop after the aphids enter into a gall. Why do wax glands not exist in the fundatrix and only appear after molting? First of all, wax secretion is energy consuming, but sexuales cannot feed since they have no mouthparts. A fundatrix cannot feed until it reaches a tender leaf. It is likely that a fundatrix does not invest in the production of wax glands to save energy and nutrients. Secondly, mobility is crucial for the newly hatched fundatrix. If a fundatrix cannot move from the lower part of a tree trunk to the top and feed on new leaves within 3-5 days, it will die (Liu *et al.*, 2014). A fundatrix without secreting wax at this stage may move faster and get more chances for survival than one with wax secretion. Without the protection of wax, the crawling fundatrices risk to be attacked by predators or be eroded by rains and their mortality rate would be much higher. This may explain the phenomenon that even though there are many fundatrices on a tree, only a few galls can be found later (Shao *et al.*, 2013). The feeding of a fundatrix on the tender leaf stimulates the atypical development of leaf tissue to form a gall with the aphid enclosed inside. After two or three days, the fundatrix molts and its body changes from black to light yellow, from slim to short and thick, and from no wax glands to several wax glands on the lateral lines (Fig. 2A, B, C, D). Therefore, the fundatrix after molting bears more complex wax glands, as well as their offspring (fundatrigeniae). The second instar fundatrix and its offspring (fundatrigeniae) live in a gall from May to October. Their individual number increased sharply and eventually reached several thousands within a single gall. In this closed microenvironment, humidity is saturated with a lot of honeydew produced everyday inside the gall (Wang *et al.*, 2020). Fundatrigeniae secrete a large amount of wax to cover the honeydew and prevent it from sticking together. Sometimes a large 'wax ball' is formed with honeydew inside. Smith (1999) also found that the primary role of the secreted wax of fundatrigeniae is to prevent aphids becoming contaminated by their own honeydew. The mealy wax coating of fundatrigeniae also prevents condensed water away from their bodies in an enclosed gall with saturated humidity. In conclusion, the structure of wax gland plates in each stage is closely related to their living microenvironments and activities. The presence or absence of wax glands before and after molting in the same stage is rare in insects, reflecting the strong adaptability of the horned gall aphid to environmental changes.

4.2. Classification of wax glands of the horned gall aphid

Insect exocrine glands can be classified into classes 1 and 3 according to the appearance and structural organization of the secretory cells (Noirot and Quennedey, 1974). Our results confirm that the wax glands of *S. chinensis* belong to class 1, which is made up by a single layer of epidermal cells (Smith, 1999). The secretory cells are covered by a cuticle like any common epidermis. Besides the extensive SER secreting lipids in the wax gland cells, there are also numerous strands of SER extending into the microvilli. This facilitates the transportation of the lipidic secretion into the microvilli, from where it is then released from the secretory cell. The cells also contain some locally concentrated and mostly circular accumulations of RER. This is indicative for protein production, but it remains unclear whether the wax filaments themselves contain a proteinaceous fraction. The large number of mitochondria in wax gland cells provides energy for wax secretion. The darker exocuticle overlaying the wax gland plates has a low electron density may be beneficial to wax secretion. Cells are only connected by septate junctions and do not need complex extracellular structures to provide mechanical strength. Apical microvilli increase the cell surface, allowing the gland cells an efficient uptake of precursor molecules from the hemolymph basally, and an efficient discharge of secretory products apically (Noirot and Quennedey, 1974; Billen and Morgan, 1998).

4.3. Comparsion of wax glands between the horned gall aphid and other aphids

Aphids in Adelgidae, Phylloxeridae, Aphididae: Eriosomatinae and Hormaphidinae have typical wax gland plates. Wax gland plates of aphids are highly diverse in distribution, degree of development, shape and structure (Smith 1999; Chen and Qiao, 2012). The wax gland plates of aphid species in Hormaphidinae have different arrangements, such as six rows, four rows, two rows on the dorsum and even scattered on the dorsum. They have a variety of shapes, such as rosette-shaped, band-like, chain-like, mosaic-like, elliptical, oval, round, and poly gonal. (Chen and Qiao, 2012). Similar to the aphid *Ceratovacuna silvestrii*, all stages except the newly hatched fundatrix of the horned gall aphid have six rows of wax gland plates on the dorsum, and each wax gland plate is composed of polygonal multi-facet depressions. While the wax gland plate of *C. silvestrii* is composed of rosette-shaped, multi-facet, single-facet plates represent a simple type that is present in many Hormaphidinae genera. In general, wax gland plates in Hormaphidinae appear to have evolved towards degeneration (Chen and Qiao, 2012). They have changed from being distributed in rows to being scattered over the dorsum, reduced in number from six to four and finally to two rows, and from being distributed on all segments to just one segment. Therefore, like *C. silvestrii* in Hormaphidinae, six rows of wax gland plates on the dorsum of the horned gall aphid appear to be the primordial type of wax gland plates in aphids (Chen and Qiao, 2012).

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Conflicts of Interest: The authors declare that they have no conflicts of interest to this work.

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