Evolutionary scenarios for unusual attachment devices of Phasmatodea and Mantophasmatodea (Insecta)

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Structure and Function of the Arolium of Mantophasmatodea (Insecta)

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ABSTRACT All species of the insect order Mantophasmatodea characteristically keep the 5th tarsomere and pretarsus (arolium plus two claws) turned upwards and off the substrate. The unusually large arolium was studied in two species of Mantophasmatodea using bright field light microscopy, reflection microscopy, fluorescence microscopy, TEM, SEM, and Cryo-SEM. It contains an epithelial gland, numerous tracheoles, and nerves. The gland consists of enlarged epithelial cells with large nuclei, mitochondria, RER, golgi complexes, microtubules, and numerous secretion vesicles. Evidence for exocytosis of the vesicles into the gland reservoir between the epithelial gland and the thick cuticle could be observed. Cryo-SEM revealed that the ventral side of the arolium and distal part of its dorsal side are covered with a liquid film. Fluid footprints of arolia of individuals walking on a glass plate also indicate the presence of secretory fluid on the arolium surface. Behavioral experiments using animals with ablated arolia showed that representatives of Mantophasmatodea do not need their arolia to detect and respond to vibratory communication signals nor to catch small to medium-sized prey. Individuals with ablated arolia were not able to move upside down on a smooth glass plate. We conclude that Mantophasmatodea use their arolia for attachment when additional adhesion force is required (e.g. windy conditions, handling large prey, mating). They can bring their arolia in contact with the surface in a very fast reflex (18.0 ± 9.9 ms). The secretory fluid found on the surface is produced by the gland and transported to the outside, presumably through small pore channels, to enhance adhesion to the substrate. J. Morphol. 270:1247–1261, 2009.

KEY WORDS: Mantophasmatodea; arolium; attachment device; gland; ultrastructure

INTRODUCTION

In May 2002, the description of the insect order Mantophasmatodea (Klass et al., 2002) created considerable scientific interest. Mantophasmatodea (heelwalkers) were initially described from two old preserved specimens from Namibia (*Mantophasma zephyrum*) and Tanzania (*Tanzaniophasma subsolanum*; Klass et al., 2002). Extant species were subsequently reported from South Africa (Picker et al., 2002), where the majority of the 20 known extant species occurs (Klass et al., 2003; Damgaard et al., 2008). All known heelwalkers are small to medium sized, apterous predators. They are easily recognized by their unusual habit of keeping the 5th tarsomere and enlarged pad (arolium) of all legs permanently raised without contact to the substrate; however, the function of this unique behavior is still unknown.

The insect arolium mainly serves as an attachment structure enabling them to walk on vertical surfaces and ceilings, and is probably a derived groundplan feature of Neoptera, (Beutel and Gorb, 2006, 2008). As defined by Beutel and Gorb (2001, after Dashman, 1953a) it is a median hollow pretarsal lobe, which can be completely membranous or at least partly sclerotized. It usually possesses a smooth or slightly microstructured surface and its cuticle consists of loosely packed fibers oriented perpendicularly or at a certain angle to the surface (Gorb, 2008). Arolia are found in many insect

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groups: Plecoptera (Beutel and Gorb, 2001); Dermaptera (Günther and Herter, 1974; Haas and Gorb, 2004); Blattodea (Roth and Willis, 1952; Roth, 1991); Isoptera, Mastotermitidae (Watson and Gay, 1991); Orthoptera, Caelifera (Rentz, 1991; Perez Goodwyn et al., 2006); Phasmatodea (Key, 1991; Beutel and Gorb, 2008); Hemiptera, Sternorrhyncha (psylloids), Auchenorrhyncha (Frantsevich et al., 2008), and some Heteroptera (Carver et al., 1991); Neuroptera (Beutel and Gorb, 2001); Hymenoptera (Naumann, 1991; Baur and Gorb, 2000; Federle et al., 2001; Frantsevich and Gorb, 2002, 2004); Trichoptera (Beutel and Gorb, 2001); Lepidoptera (Nielsen and Common, 1991); and Diptera, Tipulidae (Hennig, 1973; Gorb, 2001). The cuticle of the arolium of at least some groups of insects consists of natural friction-active materials with a specific inner structure (Beutel and Gorb, 2001). However, the anatomy and ultrastructure have rarely been investigated (e.g. Slifer, 1950; Scholz et al., 2008).

The arolium of Mantophasmatodea is larger and more complex than in almost all other insect groups. Beutel and Gorb (2006, 2008) published the first description of the attachment structures of *Mantophasma zephyrum*. They emphasized that the arolium is only lowered onto smooth surfaces and then in emergency cases, when it is brought in contact with the substrate with a very fast reflex. They also described euplantulae on tarsomeres 1–4, and acanthae on the surface of both types of attachment structures, which are similar to those found in *Timema nevadense* (Phasmatodea), but unknown among other groups of insects (Beutel and Gorb, 2006, 2008).

In this study, we provide a first detailed morphological and ultrastructural investigation of the tarsus and pretarsus of two mantophasmatodean species using different techniques such as bright field light microscopy, reflection microscopy, fluorescence microscopy, transmission electron microscopy (TEM), scanning electron microscopy (SEM), and Cryo-SEM. High-speed camera was applied to visualize fast movements of the arolium during attachment and detachment. Additionally, behavioral experiments were carried out, to test for various hypothesized functions of the arolium for (i) receiving vibrational signals, (ii) identifying and catching prey, and (iii) attachment on smooth surfaces, by using two groups of individuals with and without ablation of their arolia of all legs. Finally, we discuss possible functions of the arolium gland in terms of functional morphology and implications on the behavior of Mantophasmatodea.

MATERIALS AND METHODS

Investigated Species

Specimens of *Karoophasma biedouwense* Klass et al., 2003 (Austrophasmatidae) were collected in August 2007 as nymphs or adults at Clanwilliam Dam (Western Cape Province, South Africa, 32.21°S; 18.88°E), where they occur in Olifants Sandstone Fynbos vegetation in the arid parts of the Fynbos biome. Specimens of *Mantophasma kudubergense* Zompro and Adis, 2006, (Mantophasmatidae) were collected in March 2006 in the Erongo Mountains (Namibia, 21.73°S; 15.80°E). Individuals were reared separately in plastic pots (height 50-90 mm, diameter 55–95 mm) under constant environmental conditions (22°C, 25% RH). Vestigial fruit flies (*Drosophila* sp.) were provided at least every second day. Water was supplied by a saturated wad of paper towel.

Light microscopy. Adult male *K. biedouwense* ($n = 5$) were immobilized by chilling, immediately fixed in alcoholic Bouin's solution for 1–2 days, and then stored in 70% ethanol. Dissected parts (legs) were dehydrated in an ascending ethanol series and embedded in Agar Low Viscosity Resin under vacuum impregnation. Of the distal ends of two forelegs, two midlegs and one hindleg, serial semithin sections (1 μm thick, ~400–800 sections per leg) were made using an ultra microtome Leica EM UC6 with diamond knives. The sections were stained with a mixture of 1% azure II and 1% methylene blue in an aqueous 1% borax solution (diluted 1:20 in aqua bidest) (Richardson et al., 1960) for ~15 s at 70°C. An Olympus CX41 microscope equipped with an Olympus E330 digital camera was used for analysis of the semithin sections and production of micrographs.

For fluorescence microscopy, tarsi of two *M. kudubergense* were cut off, mounted on cover-slips in a water-soluble medium (Moviol), and observed in a fluorescence microscope (Zeiss Axioplan) in one of three bands of wavelengths: green (excitation 512–546 nm, emission 600–640 nm), red (excitation 710–775 nm, emission 810–890 nm), and UV bands (excitation 340–380 nm, emission 425 nm). Insect cuticle has a strong autofluorescence at a variety of wavelengths from the blue-green area to deep-red. However, resilin, an elastic protein of the cuticle, is autofluorescent at a very narrow band of wavelengths (ca. 400 nm) and has been previously reported from different mechanical systems of insects (Andersen and Weis-Fogh, 1964; Gorb, 1999). This specific property of the protein allows for its detection in biologically native structures without immune labeling or other treatments.

To visualize real contact area between the arolium and the glass substrate, and to prove the presence of fluid footprints in living animals standing on a glass ceiling, the destructive interference of reflected white light in the glass-arolium interface was used. *M. kudubergense* males and females ($n = 3$) were examined under an Olympus reflection microscope while walking on a Petri dish. Images were taken from single arolia of animals standing on the glass ceiling. As soon as the contact was interrupted, the footprint was photographed at the site of the previous contact.

Transmission electron microscopy. The animals were dissected and the arolia of three males fixed in Karnovsky mixture (2% paraformaldehyde, 2.5% glutaraldehyde in 0.1 M cacodylate buffer) for 2.5 h, a mixture of Karnovsky and Tannin (1%) for 45 min, and in a 1:1 mixture of 0.1 M cacodylate buffer and an aqueous 1% solution of OsO_4 for 2 h. Fixed parts were dehydrated and embedded in Agar Low Viscosity Resin, and serial ultrathin sections (60–90 nm thick) were made as described above. Sections were stained with uranyl acetate (30 min) and lead citrate (10 min) and examined with a Philips EM 208 transmission electron microscope.

Scanning electron microscopy. Three male and two female *K. biedouwense* were anesthetized by chilling and immediately fixed in alcoholic Bouin's solution for 1–2 days. Dissected parts (legs) were dehydrated in ascending ethanol series and dried with hexamethyldisilazane (HMDS) for 30 min. The material (front, middle, and hindlegs) was placed on aluminum stubs using double stick carbon tape and silver glue, sputter coated with gold and examined with a Philips XL20 scanning electron microscope.

Measurements of all structures were done after fixation and dissection of the investigated material.

Cryo scanning electron microscopy. Male and female *M. kudubergense* ($n = 3$) were anesthetized by chilling. Excised legs were mechanically clamped in the sample holder. The sample was frozen in liquid nitrogen, transferred to the cryostage of the preparation chamber (−140°C), and then sublimated at a temperature of −90°C for 3 min, to remove contamination by condensed ice crystals. The sample was coated in the frozen condition with gold-palladium (3 nm thickness) and observed at $t = -120$°C and an accelerating voltage of 1–3 kV in a Hitachi 4800 SEM. We used the instrument's lower SE detector, which is capable of excellent resolution of the very small differences in the substrate surface profile, in order to detect fluid droplets on the surface.

Ablation Experiments

To test the function of the arolium in male *K. biedouwense*, ablation experiments were carried out. For the "cut group", eight males were immobilized by chilling, held tight between two pieces of plastic "cling" film, and the arolia of all legs carefully cut off with a scalpel-blade. For the "control group", eight males were handled in exactly the same way as the "cut group", but without the ablation of the arolia. Two experiments were conducted: (i) a playback experiment to investigate the ability of males to detect and respond to the vibratory call of a conspecific female (Eberhard and Picker, 2008) with and without arolia, and (ii) an attachment experiment to investigate the ability of the two groups to stand and run on a smooth surface. Furthermore, the ability of *K. biedouwense* to catch prey with or without arolia was observed. Because the insects use their legs (especially fore- and midlegs) for prey capture, the use of the enlarged arolia for catching prey should be tested. The arolium also has a robust row of setae which might facilitate prey capture.

Playback experiment. Mantophasmatodea have been shown to use species-specific substrate vibrations, produced by tapping their abdomen onto the substrate, for mate location and recognition (Eberhard and Picker, 2008). The legs of communicating insects are in direct contact with the substrate and, not unexpectedly, are the sites of most sensitive substrate-borne vibratory receptors (Čokl et al., 2006). Using the ablation experiment, the significance of the distal tarsus and arolium for receiving and reacting to conspecific vibrational signals was tested. All males of both groups were tested once on the same day before the ablation-treatment. Five days later, the ablation for the "cut group" and the ablation-treatment for the "control group" was performed. On the following day, the playback experiment was repeated with both groups and differences in the reaction of the individuals to the playback were investigated.

For the playback experiment, the same experimental setup (the "Y"-system) employed in Eberhard and Picker (2008) was used. Each test lasted for 10 min. A single male was placed onto the vertical stem of the "Y" system and left there to settle for 1 min. Prerecorded vibratory signals from a *K. biedouwense* female were used as vibrational stimulus and applied to either the left or the right branch of the "Y" system via a loudspeaker (NWX-5035-8SQ, low-midrange loudspeaker, impedance 8 Ω, Nippon America Co., Atsuta, Nagoya, Japan), switching was done randomly at intervals. Vibrational responses of males were monitored through the membrane of a lapel microphone (Joseph® J-1102B), which was connected to a portable cassette recorder (Marantz, PMD430). Recorded signals were digitized (44,100 Hz sampling rate, 16-bit sample size, mono) and stored on a PC using CoolEditPro software (Version 2.1, Syntrillium Software Corporation, USA). All tested individuals were monitored on video with a Canon MVX40 video camera (Canon Inc., Japan). During trials 10 different response parameters of test males were scored (see Table 1). The test was terminated when the individual had arrived at the source of stimulation (the loudspeaker), or after 10 min, when the loudspeaker had not been located. All playback experiments were carried out between 08 h00 and 17 h00 in a quiet room at room temperature (~20°C). Each male was tested only once before and once after the ablation-treatment.

Chi square tests were used to compare differences in the number of males responding (as indicated by calling, abdominal movement, or searching behavior) to the one-sided stimulation, as well as to compare differences in their ability to locate the source of stimulation before and after ablation of the arolium. A nonparametric Mann-Whitney U test was used for pairwise comparison of pulse train rate, searching distance, and latency times of responses (abdominal rubbing, calling, and searching) in the different groups (comparison of "cut group" before and after ablation, and comparison of "cut group" and "control group").

Attachment experiment. An unscratched glass plate was used to test the two groups of *K. biedouwense* males in their ability to attach to smooth surfaces. In a first trial, the glass plate was placed in an angle of 90° to the floor. A male was put onto the plate and its grip onto the plate recorded. After 30 s the male was stimulated to run on the surface by pushing it gently from behind, and its ability to remain attached noted. In a second trial, the glass plate was orientated horizontally, and the insects placed on its lower surface. Again it was recorded if the male could hold fast and/or run on the smooth surface. A Fisher's exact test was used to detect differences between the "cut-" and "control group" in the four test situations (90° stand, 90° run, upside down stand, upside down run).

In addition, video recordings of *M. kudubergense* feeding on large prey (flies: *Calliphora vicina*) were conducted in different behavioral situations, such as upside down position on the ceiling, wall position, and regular position on the flat substrate. Single frames were qualitatively used for further analysis.

High Speed Video Recordings

To obtain information about terminal tarsomere movements during contact formation of the arolium with the substrate, *M. kudubergense* males were placed in a large plastic Petri dish (ø = 190 mm), strongly locally illuminated with a fiber optical light source, and video recorded with a high-speed camera Kodak Motion Corder Analyzer PS-220 at frequencies of 1,000–3,000 fps. The recordings were performed from above, below, and from one side. The high-speed video sequences allowed us to estimate the time required for the arolium to make contact with the substrate. Attachment and detachment times were estimated from the high-speed video recordings starting with the frame where the initial relative movement of the terminal tarsomere in comparison with the previous frame was visible, and ending with the frame, where no relative change in movement was observed. Because attachment and detachment of the arolium are controlled by the motion of the terminal tarsomere, both attachment and detachment times were estimated for the entire terminal tarsomere, not just for the arolium. Information about the speed of tarsus movements was obtained from sequences of digital images and Adobe Premiere 5.1 software.

RESULTS

General Morphology

The tarsi of *K. biedouwense* and *M. kudubergense* are five-segmented with the first four tarsal segments bearing euplantulae with specialized hairy attachment structures (Beutel and Gorb, 2008). Tarsomere 5 is elongate and thin compared with the proximal four tarsomeres. The pretarsal structures, i.e. the large arolium, the paired lateral claws, and the unguitractor plate are attached to it. Tarsomeres 5 of all legs, together with the

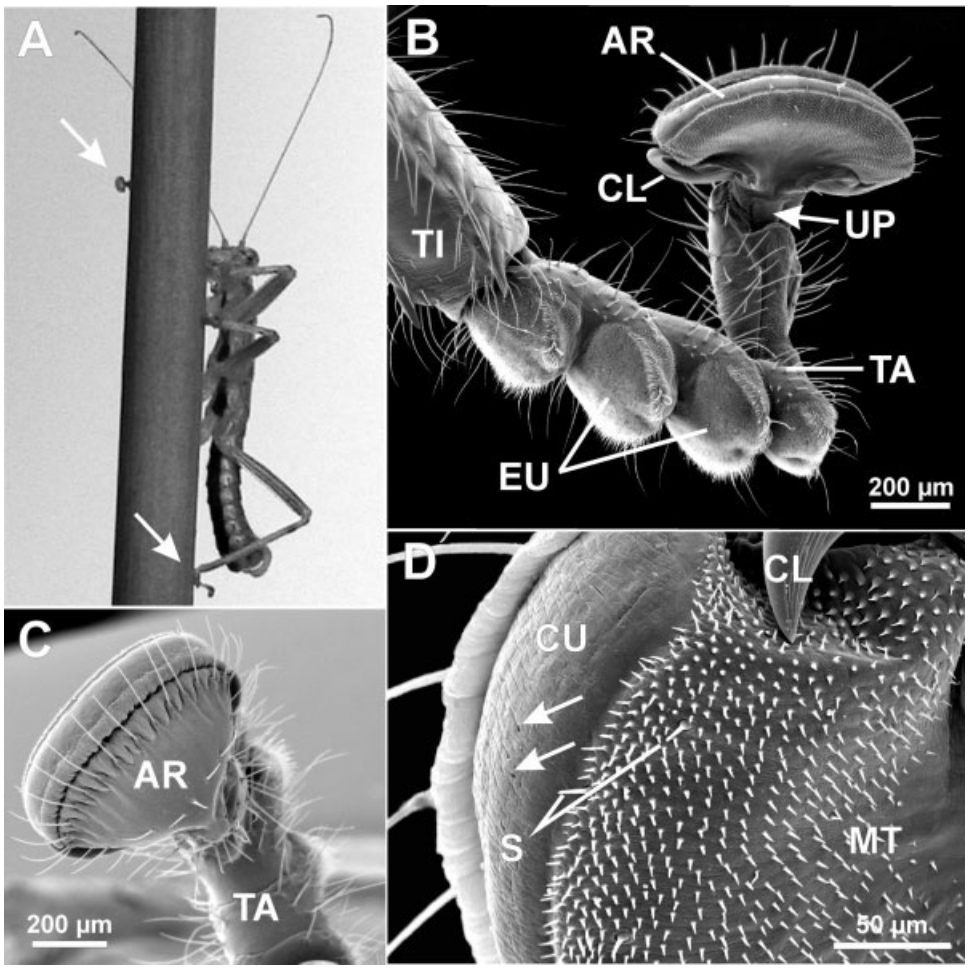

Fig. 1. **A**: *Karoophasma biedouwense* male in usual walking or resting posture with uplifted 5th tarsomere and arolium (white arrows). **B:** Scanning electron micrograph of tarsus and pretarsus of *K. biedouwense*. Tarsomeres 1-4 bear hairy euplantulae while tarsomere 5 is held upright to keep the arolium together with the two lateral claws off the substrate. An unguitractor plate is also visible. **C:** Dorsal side of the arolium. Regularly spaced long setae cover the margin of the dorsal side; additionally there are some shorter setae on the dorsal surface. Note the soft cuticle on the distal margin of the arolium. **D:** Detail of the distal ventral side of the arolium bearing microtrichia together with trichoid sensilla. Note some "pores" within the soft cuticle at the distal margin of the arolium (arrows). AR, arolium; CL, claw; CU, cuticle; EU, euplantulae; MT, microtrichia; S, trochoid sensilla; TA, tarsus; TI, tibia; UP, unguitractor plate.

arolia, are usually raised and kept distant from the substrate in the resting position and during walking (Fig. 1A, 7A, 8C,D). The dorsal side of the arolium bears a row of 15–18 regularly spaced long trichoid sensilla (140–180 µm long) along the distal margin (Fig. 1C, 3A, 5D), and in addition 10–12 trichoid sensilla of variable length (80–160 µm) dispersed on the dorsal surface (Fig. 1C). The ventral side is covered with tiny microtrichia (about 5 µm long) and some small trichoid sensilla of ~10–15 µm length (Fig. 1D, 5A–D). The dorsal and ventral cuticle of the arolium is largely sclerotized; only the laterodistal margins consist of flexible cuticle, which is characterized by a more or less regular pattern of surface corrugations, mainly consisting of longitudinal ridges (Fig. 1D). The cuticle of this distal part is thickened (up to 200 µm), and consists of a fibrous material with a network of threads arranged at an angle to the surface (Fig. 2A,B,D,E). Fluorescence microscopy has demonstrated that the pretarsus and its rigid parts (unguitractor plate and claws) are elastically suspended on cuticular structures rich in resilin (see Fig. 3).

The cuticle of the arolium is internally lined with an epidermis which is ~10 µm thick. Toward the distal region of the arolium, the epidermis is thicker (about 30–50 µm), greatly folded and detached from the cuticle, thus forming a gland with unknown function (see Fig. 2). There were no obvious differences between the glands within the arolia of all three leg pairs of male and female *K. biedouwense*. The space between the glandular epithelium and the cuticle forms a gland reservoir. No distinct openings of the glands to release their secretions could be detected; however, some

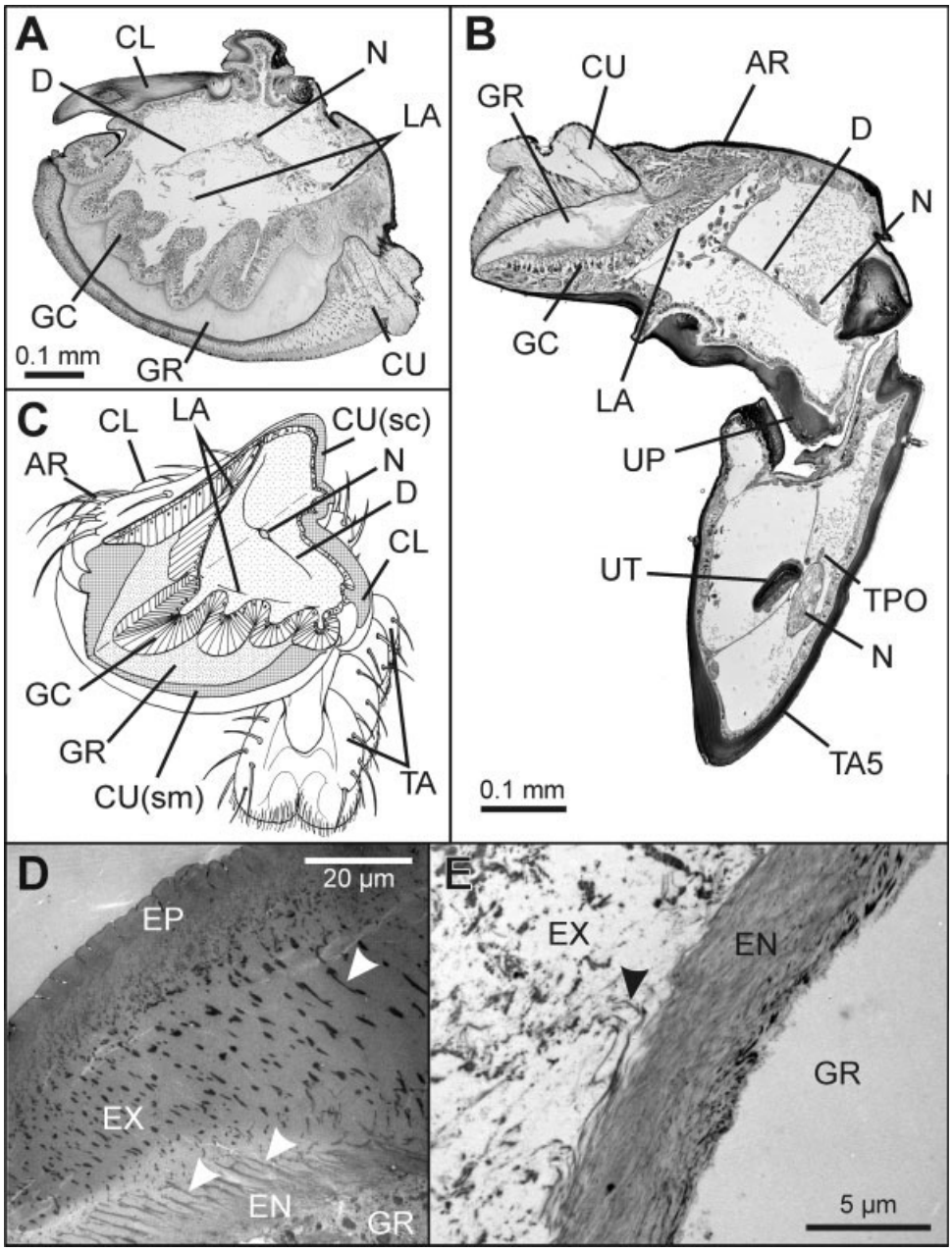

Fig. 2. *Karoophasma biedouwense*, semithin sections, TEM, and schematic drawing of the tarsus and arolium. **A:** Horizontal section of arolium. The glandular epithelium is detached from the thick cuticle with the space between the two structures forming a gland reservoir. **B:** Sagittal section of arolium and tarsomere 5. **C:** Schematic drawing of the tarsus and pretarsus of *K. biedouwense*. **D:** Transmission electron micrograph of the thickened, smooth cuticle at the laterodistal margin of the arolium. It consists of a network of threads arranged at an angle to the surface (arrowheads). **E:** Detail of basal part of the arolium cuticle. AR, arolium; CL, claw; CU, cuticle; CU(sc), sclerotized cuticle, CU(sm) smooth cuticle; D, diaphragm; EN, endocuticle; EP, epicuticle; EX, exocuticle; GC, gland cells; GR, gland reservoir; LA, lamina; N, nerve; TA, tarsus; TPO, tarso-pretarsal scolopidial organ; UP, unguitractor plate; UT, unguitractor tendon.

"pores" (0.40–0.70 µm in diameter) within the longitudinal ridges of the smooth cuticle at the lateral and distal sides of the arolium were found (Fig. 1D).

The hemolymph space of the arolium is divided into two hemolymph chambers by a cellular diaphragm (2–5 µm thick) which opens on the lateral sides (see Fig. 2). The diaphragm consists of highly interlocked cells and is covered with external laminae; it is attached to the proximal epidermis of the arolium by some strands of connective tissue. It also contains a number of tracheoles and small nerve branches. A leg nerve innervating the arolium branches distally to supply the sensory setae

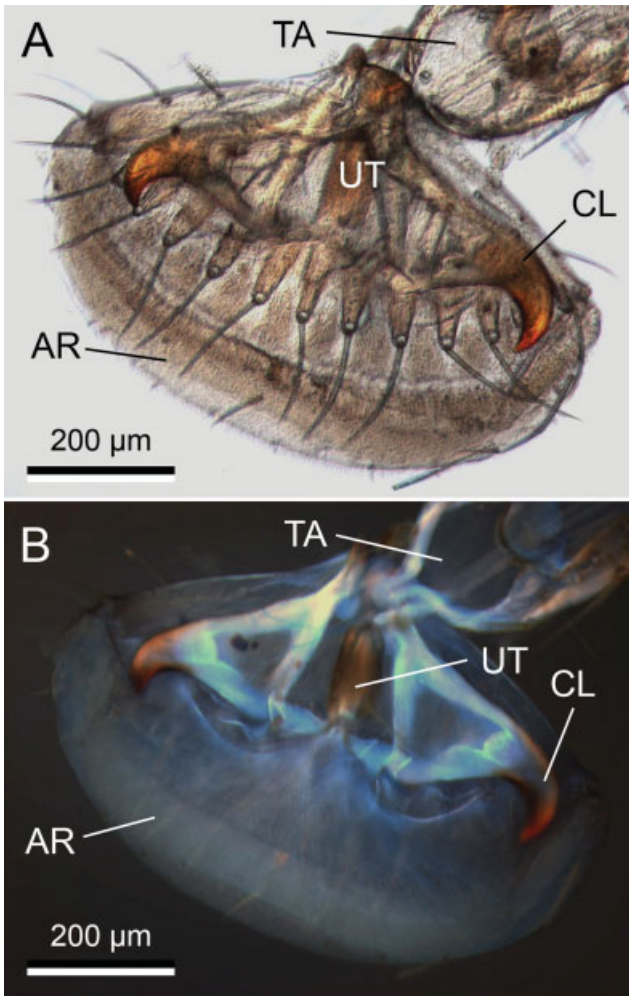

Fig. 3. *Mantophasma kudubergense*, freshly cut off whole mounted arolium in bright field (**A**) and fluorescence mode (**B**) of light microscope. In the fluorescence mode, three images taken at different wavelengths were superimposed to show different autofluorescence of cuticular structures. Reddish and yellowish areas correspond to the tanned cuticle, whereas bluish ones are those containing resilin. AR, arolium; CL, claw; TA, tarsus; UT, unguitractor plate.

on the dorsal and ventral surfaces. The nerve (20 μm in diameter when entering the arolium) is always in close proximity to a trachea (about 18 μm in cross section when entering the arolium) and is embedded in the diaphragm. A tarso-pretarsal scolopidial organ is present in tarsomere 5; it arises from the leg nerve at about the middle of the tarsomere (Fig. 2B) and attaches dorsally to the arolium. A schematic drawing of the arolium and arolium gland is shown in Figure 2C.

Gland Structure

The tissue of the arolium glands consists of high prismatic epidermal cells. The glandular epithelium is strongly folded, the folds occupy up to half of the lumen of the arolium (about 200 μm in cross section). It is kept separate from the hemocoel by a thin continuous acellular basal lamina (approximately 0.2–0.5 μm thick) that contains bundles of elastic fibers. Additionally, at the proximodorsal region of the gland and above the thin acellular lamina, a thick noncontinuous lamina is present (0.7–2.5 μm thick). It consists of fibrous, electron dense material (Fig. 4A) and extends from the dorsal margin of the gland to its ventral third at the region of the epithelial folds; it does not follow the course of the folded tissue but remains straight; therefore, only the basal part of the gland (the basal margins of the epithelial folds) is attached to this lamina (see Fig. 2). The cell's apical sides do not exhibit a lamina to keep the gland separate from the secretion storing reservoir.

The nuclei of the glandular epidermis are large, round-oval (~3 to 5 μm in diameter or up to 9 μm long when oval), and mainly located in the basal half of the cells; they contain only small amounts of heterochromatin (Fig. 4A,B). The gland cells contain numerous large, mostly elongated mitochondria, an extensive rough endoplasmic reticulum (RER), golgi complexes (Fig. 4B), and secretion vesicles of different shape and contrast. Few microtubules are visible within the cells (Fig. 4B). On the basal side of the gland cells there are evaginations of the basal membrane; the cells are connected to the extracellular matrix by hemidesmosmes (Fig. 4A). On their apical side, the gland cells are connected to each other by zonulae adhaerentes that lie above pleated septate junctions (Fig. 4C). On the apical side of the cells there are numerous microvilli. At the base of these microvilli, exocytosis of electron dense, coated vesicles into the gland reservoir could be observed (Fig. 4C,D).

Fluid

Cryo-SEM data on freshly frozen arolia showed that the ventral side and the distal part of the dorsal side are covered with fluid (Fig. 5B,C,E,F). The dorsal side contains grooves, which in the fresh arolium are filled with the fluid. The exact origin of the fluid remains unknown.

In *M. kudubergense* the contact with the substrate is made primarily by the distal rim of the arolium (Fig. 6A,B), where the cuticle is flexible. In some cases, additionally more proximal regions (those covered by microtrichia) were involved in contact formation (Fig. 6C). After the arolium had lost its contact with the glass, fluid footprints were observed on the glass surface indicating that some kind of secretion is spread within the contact (Fig. 6D,E). Interestingly, residues of the fluid form parallel lines of droplets, whose orientation corresponds to the orientation of the cuticular folds of the soft cuticle at the distal rim of the arolium (Fig. 6E).

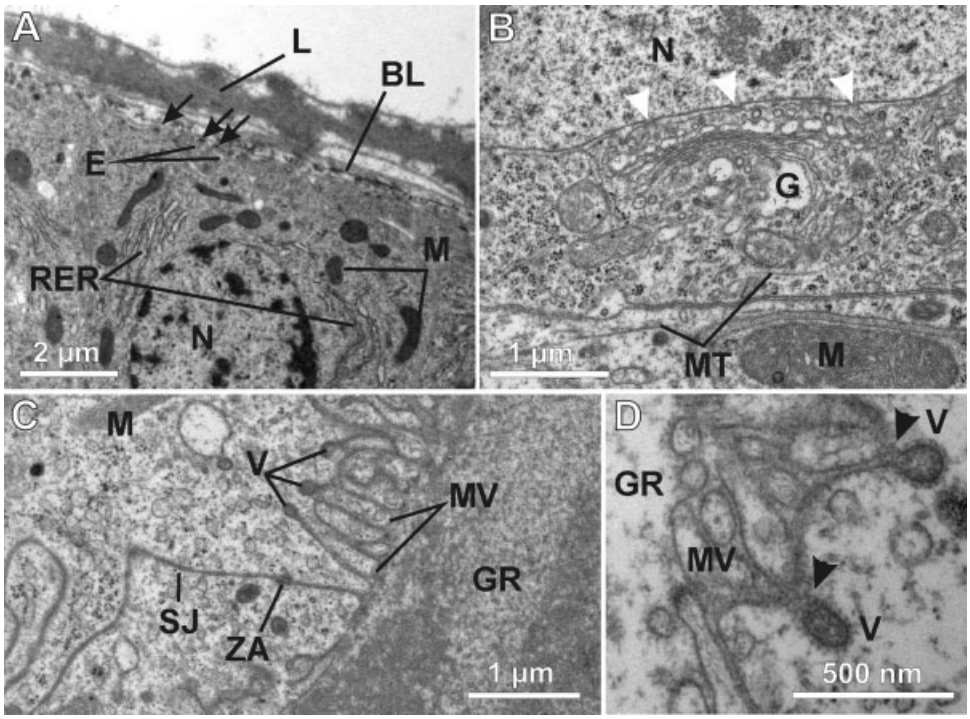

Fig. 4. *Karoophasma biedouwense*, TEM of epithelial gland cells within the arolium. **A:** Basal side with evaginations of the basal cell membrane (E) and hemidesmosomes (black arrows) that connect the gland cells to the extracellular matrix. Note the thick lamina (L) above the basal lamina (details see text). **B:** Organelles within cells. Note the nuclear pores that allow for the transport of water-soluble molecules across the nuclear envelope (white arrowheads). **C, D:** Apical side; the cells are in close contact to each other by septate junctions (SJ) and zonulae adhaerentes (ZA). Exocytosis of coated vesicles at the base of the microvilli into the gland reservoir can be seen (black arrowheads). BL, basal lamina; E, evaginations of basal cell membrane; G, golgi complex; GR, gland reservoir; L, lamina; M, mitochondria; MT, microtubules; MV, microvilli; N, nucleus; RER, rough endoplasmic reticulum; SJ, septate junction; V, vesicles; ZA, zonula adhaerens.

High-Speed Video Recordings

Behavioral observations on *M. kudubergense* showed that animals usually walked even on vertical smooth walls and hanging from ceilings without using their arolia (Fig. 7A, 8C,D). However, in situations with drastically increased mass (e.g. during manipulation of a large prey item as in Fig. 8A,B, females carrying males in copula, or females fully packed with eggs), arolia were regularly used, at least on the ceiling. Similarly, at strong wind pulses and substrate vibrations, insects immediately secured their attachment by bringing their arolia down in contact with the substrate (Fig. 7B, 9).

Using high-speed video recordings of 28 sequences of attachment and 16 sequences of detachment, attachment times of 6–43 ms were obtained, whereas detachment time was 11–191 ms (average and SD are 18.0 ± 9.9 ms and 49.3 ± 50.3 ms, respectively; Fig. 9).

Ablation Experiments

Playback experiment. The playback experiment was conducted in order to test whether the distal tarsus and arolium, especially the tarso-pretarsal scolopidial organ, are involved in the detection of substrate vibration. The one-way playback trials showed clearly, that males without arolia reacted exactly the same way as males with arolia in all response variables tested. No differences were found for all measured parameters between the "cut group" before and after ablation or between the "cut group" and the "control group" after the ablation-treatment.

Before the start of the playback, all males placed on the stem of the "Y" system remained in this position until the stimulus started. Means of the measured reactions for the groups before and after the ablation-treatment are shown in Table 1. No significant differences were found between all groups for the occurrence of calling and for the location of the source of stimulations ($P > 0.05$ for both parameters, Chi square test). For abdominal rubbing and searching, no statistical test was computed because all tested individuals reacted positively, thus being the same for every test (100% of individuals reacted positively in each group before and after ablation-treatment). Pairwise comparison of latency times, pulse train rate, and searching distance showed no significant differences

TABLE 1. Playback experiments with 2 groups of K. biedouwense *males receiving calls from a conspecific female*

Group	Abdominal rubbing	Abdominal rubbing latency [s]	Calling response	Calling latency [s]	Pulse train rate per min	Searching	Searching latency [s]	Searching distance [cm]	Location of source	Start - end time [min]
Cut group before ablation	100%	16.31 ± 18.26	75%	19.00 ± 20.64	10.07 ± 4.71	100%	29.13 ± 16.45	55.63 ± 37.87	62.50%	4.28 ± 2.53
				$n = 6$	$n = 6$					$n = 5$
Control group before ablation	100%	8.71 ± 4.46	100%	18.84 ± 9.12	8.09 ± 4.88	100%	22.00 ± 19.38	65.13 ± 39.16	75%	4.10 ± 2.70
	$n = 7$	$n = 7$								$n = 6$
Cut group after ablation	100%	6.63 ± 3.25	100%	16.95 ± 18.87	11.22 ± 4.33	100%	18.25 ± 12.12	82.38 ± 49.27	100%	3.44 ± 3.08
Control group after ablation-treatment	100%	8.43 ± 9.68	100%	19.90 ± 15.54	11.25 ± 4.10	100%	20.50 ± 17.17	132.63 ± 150.76	75%	2.31 ± 1.18
		$n = 7$								$n = 6$

The cut- and control group were tested twice, once before the ablation of the arolia and once after the ablation-treatment. The test was terminated when the male arrived at the loudspeaker (source of vibration) or after 10 min. Data given as means ± standard deviation. If not stated otherwise, $n = 8$.

between the two groups after ablation, and the "cut group" before and after the ablation, respectively ($P > 0.05$, Mann-Whitney U test). Almost all tested individuals were able to locate the source of vibration (the loudspeaker) within more or less the same amount of time (no significant differences between the groups), irrespective of the presence or absence of arolia (Table 1).

Attachment experiment. All males of the "control group" (with arolia) were able to stand and run on the 90° glass plate as well as to stand and run upside down on the smooth surface. All eight males of the "cut group" (without arolia) managed to stand and run on the 90° plate, five of them could hold fast upside down on the glass plate (no significant difference to the "control group", $P = 0.20$, Fisher's exact test). Only one male of the "cut group" was able to walk upside down, whereas the other seven individuals immediately fell off after starting to move. Accordingly, the "cut-" and "control group" differed significantly when tested in their ability to run upside down ($P < 0.01$, Fisher's exact test).

Additionally, when the ability of *K. biedouwense* males with and without arolia to catch small prey (*Drosophila* sp.) was investigated for four consecutive days, no differences between the two groups could be observed; almost all individuals were able to catch all flies that were provided to them.

DISCUSSION

Structure of the Arolium and Its Gland

The homology of the specialized arolium of Mantophasmatodea with the corresponding pretarsal attachment structure found in different neopteran lineages is very likely (Beutel and Gorb, 2008). However, in its structure and performance it is quite different from the typical condition as it is found in Caelifera (Perez Goodwyn et al., 2006) and other groups of "lower Neoptera". It was pointed out in Beutel and Gorb (2008) that the strong enlargement and surfaces densely covered with acanthae suggest close phylogenetic affinities between Mantophasmatodea and Phasmatodea, with secondary modifications within the latter order. The high degree of sclerotization is another clearly derived condition occurring in both groups, whereas a largely or completely membranous arolium is almost certainly a groundplan feature of Neoptera (e.g., Plecoptera; see Gorb, 2001, 2008). The habit of raising the ultimate tarsomere with arolium under normal conditions is apparently an autapomporphy of Mantophasmatodea. The arolium of heelwalker also differs strongly from the modified, complex arolium of Hymenoptera (Snodgrass, 1956; Beutel and Gorb, 2001; Federle et al., 2001; Frantsevich and Gorb, 2002, 2004; Gladun and Gumovsky, 2006; Gladun, 2008), which consists of numerous sclerites (manubrium, arcus,

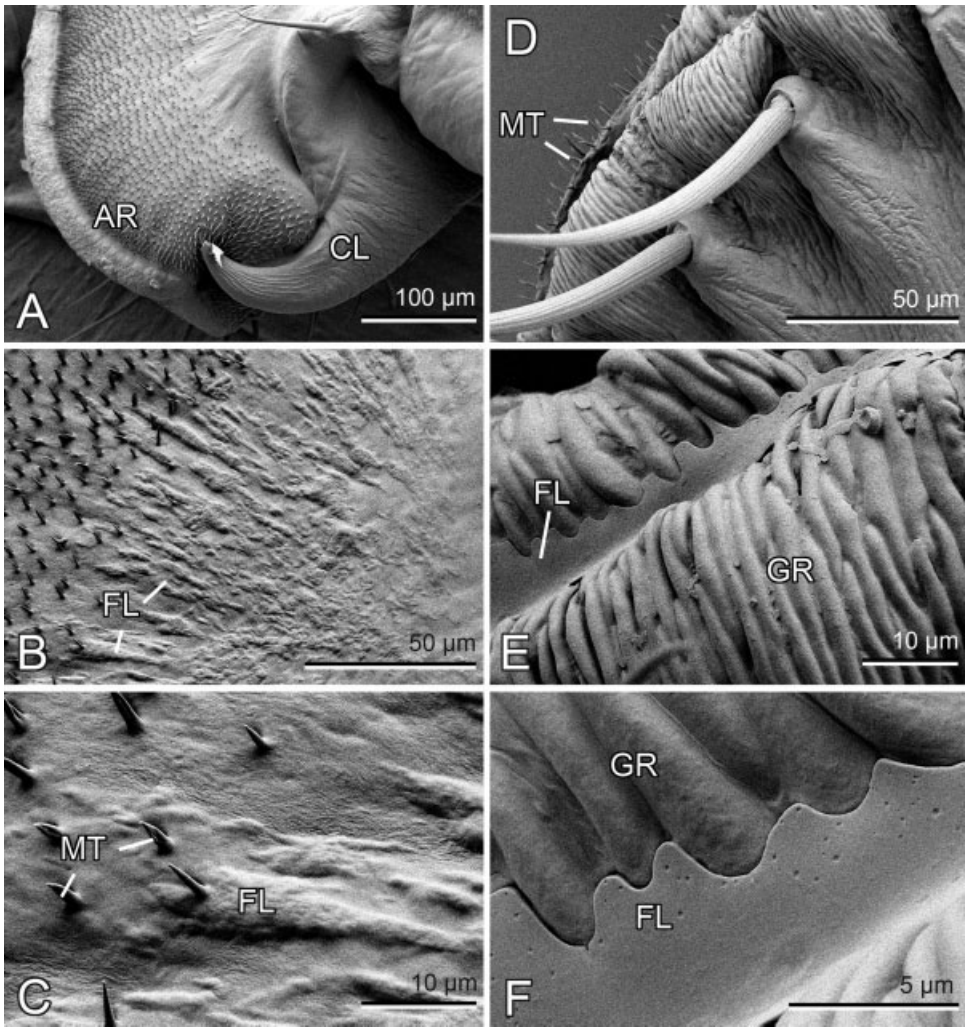

Fig. 5. *Mantophasma kudubergense*, Cryo-SEM images of the distal part of the freshly frozen arolia. **A–C:** Ventral aspect. **D–F:** Dorsal aspect. Please, note the presence of the fluid on both ventral and dorsal sides of the arolium in the vicinity of the terminal rim, which is used for initial contact formation. AR, arolium; CL, claw; FL, fluid; GR, grooves on the dorsal side of the arolium; MT, microtrichia on the ventral side of the arolium.

auxillae, planta) that are part of the mechanically complex folding–unfolding system. In the studied representatives of Mantophasmatodea, arolia are almost fully sclerotized with only the most distal margins being membranous and flexible. Only an unguitractor plate with a specialized cuticular surface covered with little cuticular outgrowths (similar to those found in other insects: Dashman, 1953b; Goel, 1972; Conde-Boytel et al., 1989; Seifert and Heinzeller, 1989; Gorb, 1996) can be distinguished from the rest of the arolium.

The arolium of Mantophasmatodea was shown to contain numerous structures such as an epithelial gland, numerous tracheae, nerves, a diaphragm, and hemolymph. These findings are in accord with Debaisieux (1938), who described the arolium of an acridid (Orthoptera); it consists of fibrous cuticle underlain by a pleated and thick epidermis, and contains hemolymph, a trachea, and a diaphragm. Scholz et al. (2008) report an epidermis covering the cuticle inside the arolium of the stick insect *Carausius morosus*; a semithin section of the arolium shows that the lumen of the arolium is filled with hemolymph. Unfortunately, no further analysis was done on the internal structure of the arolium in this study. The most comprehensive account of the anatomy of the arolium of the grasshopper *Melanoplus differentialis* was published by Slifer (1950). She described a hard pigmented dorsal side of the arolium and a soft, pliant ventral side, with an extremely thick cuticle consisting of several layers of fibrous material. It is hollow, filled with hemolymph and transversed by nerves and many tracheae. The epidermis on the ventral side consists of large cells and is somewhat detached from the thick cuticle. Slifer (1950) concluded that the primary function of this thick epidermis would be the secretion of the complex cu-

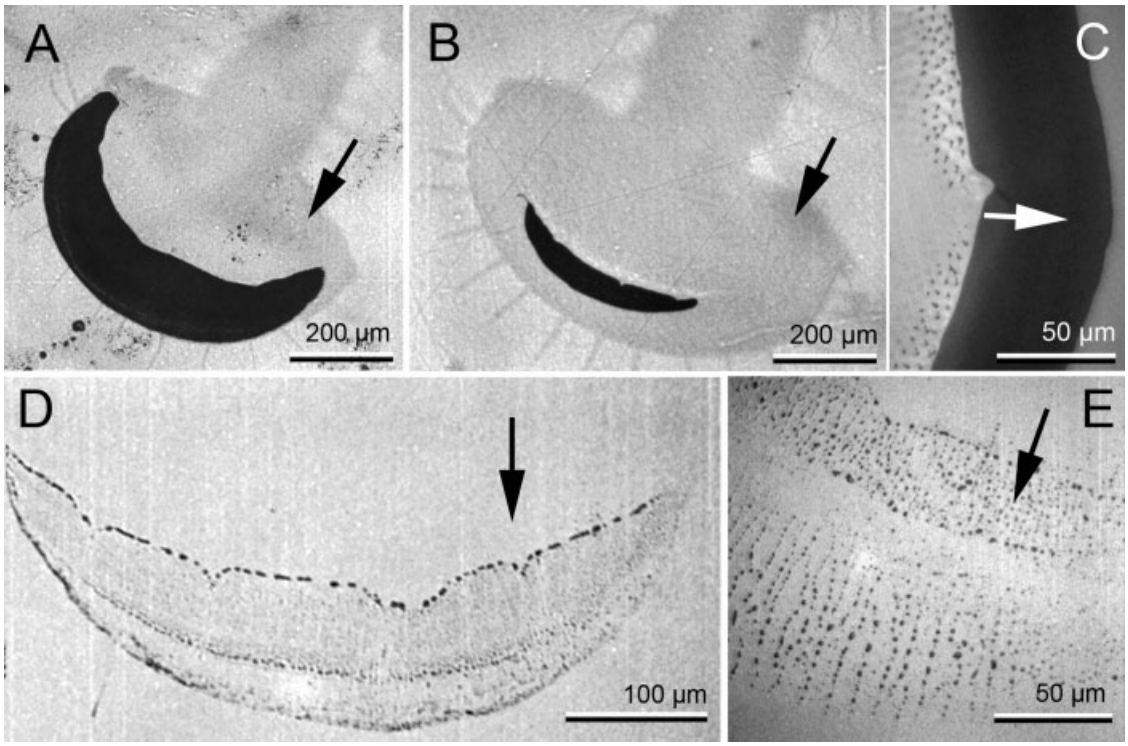

Fig. 6. *Mantophasma kudubergense*, images of arolia and their footprints obtained from living individuals standing on the glass ceiling. Destructive interference of reflected white light in the glass-arolium interface resulted in a visualization of the real contact area and allowed the detection of its changes during different stages of contact formation and breakage. **A, B:** Arolium at different stages of contact formation. **C:** Distal part of the arolium in contact (please, note that neighboring acanthae are also in full contact with the substrate). **D, E:** Arolium footprints at different magnifications. Arrow indicates distal direction.

ticle. A function to produce a fluid which permeates the cuticle was not demonstrated but assumed, even though no ducts within the arolium or footprints on a glass surface were found. The internal features described by Slifer (1950) resemble those found in Mantophasmatodea, although the arolium in this group is considerably larger and soft, thick cuticle is only present at the distal margins. In contrast, the internal structure of hymenopteran arolia differs from the mantophasmatodean arolium, as in Hymenoptera arolia lack an epidermis and other structures (Lensky et al., 1985; Federle et al., 2001; Jarau et al., 2005; Billen, 2009).

The glands within the arolia of all three pairs of legs of *K. biedouwense* consist of modified epidermal cells that can be classified as Class 1 gland cells according to Noirot and Quennedey (1974) and Quennedey (1998). The gland is likely a derivative of the epidermis; the cells are enlarged, detached from the cuticle and folded at the distal margin of the arolium at the region of the flexible cuticle. This interpretation is supported by the fact that the glandular epithelium is continuous with the more proximal epidermis of the arolium that lines its sclerotized, thin cuticle.

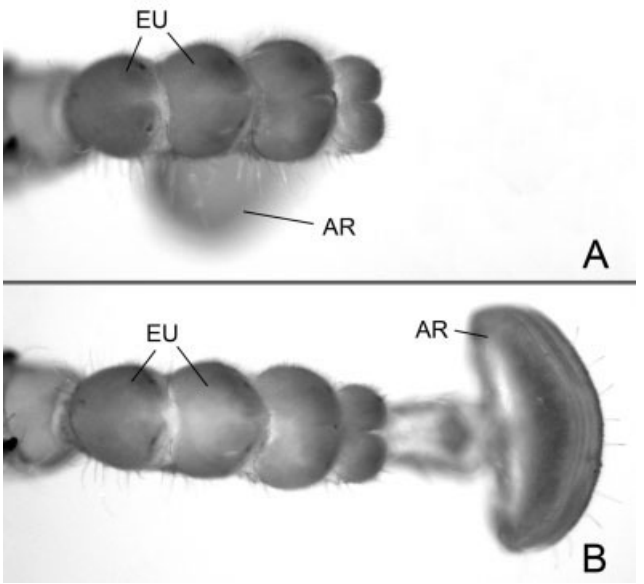

Fig. 7. *Mantophasma kudubergense*, two positions of the tarsus of living individuals standing upside down on the glass substrate (ventral aspect, binocular microscope). **A:** Euplantulae (EU) are in contact, whereas arolium (AR) is in the air. **B:** Both arolium and euplantulae are in contact.

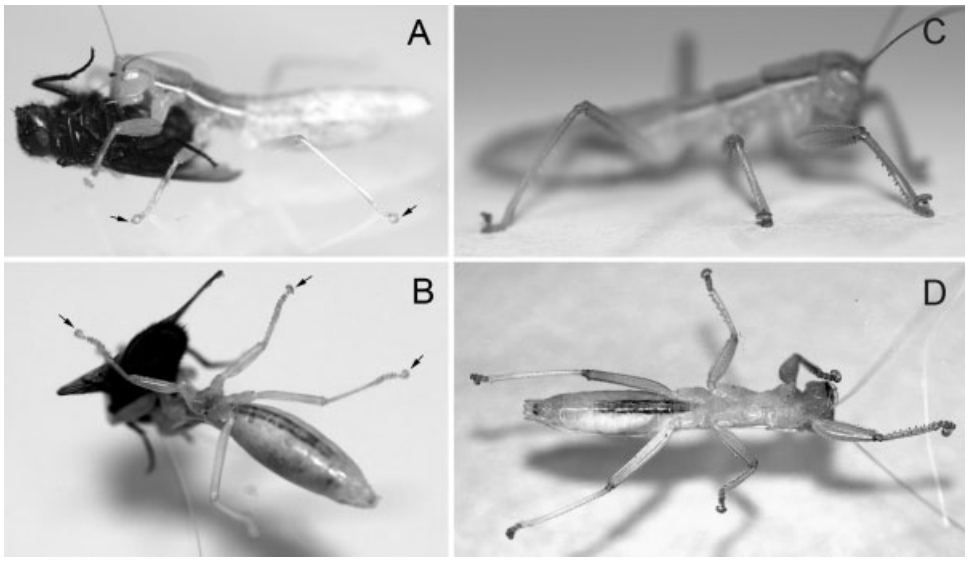

Fig. 8. *Mantophasma kudubergense* female feeding on large prey item, compared with usual posture without prey. Regular posture (**A**) and upside down posture (**B**) on the flat glass substrate with prey (arrows indicate that both arolia and euplantulae are in contact). Regular posture (**C**) and upside down posture (**D**) without prey (arolia of all legs are raised and off the substrate).

The thick lamina at the proximal region of the gland may be a second layer of basal lamina supporting the glandular epithelium and keeping the gland in place within the lumen of the arolium. Multilayered basal laminae are not unusual in insects and the second layer of the basal lamina can be widely separated from the cell surface (Ryerse, 1998). The lamina is not considered to be a connective tissue sheath, as it occurs in some exocrine glands (François, 1998), because it does not consist of a loose network of reticular fibers, microfibers, and ground structure, but rather of a definite felt-like matrix, which is a characteristic of basal laminae (Ryerse, 1998). The diaphragm situated within the arolium is regarded as the distal part of the nonpulsatile hemolymph-guiding structure found in many insects that enables a countercurrent hemolymph flow within the leg (reviews: Pass, 2000; Pass et al., 2006). In the outermost tip of the arolium the diaphragm is lacking and the two sinuses are confluent for reversion of the hemolymph flow.

The gland cells release their secretions via exocytosis into the reservoir between the gland and the thick cuticle (see Fig. 2). Evidence of exocytosis of coated vesicles into the reservoir could be observed several times in ultrathin sections (Fig. 4C,D); therefore, we suggest that the gland cells were very active at the moment of tissue fixation. It is conceivable that the secretion fluid is discharged through the "pores" that were found on the surface of the cuticle, but according to their size they could also represent sensilla homologous to those found on the euplantulae of locusts (Kendall, 1970) and cockroaches (Roth and Willis, 1952; Clemente and Federle, 2008). Another possible

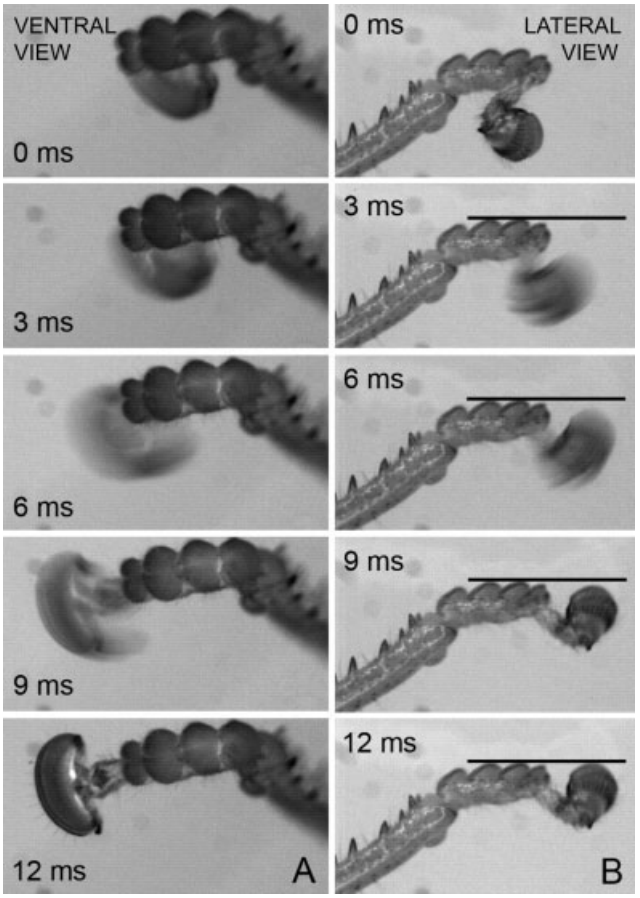

Fig. 9. *Mantophasma kudubergense* male, single frames of high-speed video recordings that show contact formation between arolium and the glass substrate of the ceiling. **A:** Ventral view. **B:** Lateral view. Time in milliseconds is indicated in each single frame. Black horizontal lines in B indicate ground level on the glass surface.

avenue of transport of fluid to the cuticular surface might be through a system of tiny porous channels, similar to the previously reported porous channels from locust euplantulae (Schwarz and Gorb, 2003). However, in semi- or ultrathin sections we could not observe channels in the thick cuticle. This was also the case in studies of Jarau et al. (2005) and Federle et al. (2001) who did not find any openings of the tarsal glands in representatives of Hymenoptera. It was therefore suggested that the arolium gland secretion remain confined within the arolium and may have a hydraulic function (Federle et al., 2001; Jarau et al., 2005; Billen, 2009). However, the occurrence and structure of pore canals among smooth adhesive pads of insects requires better documentation.

All insect adhesive pads studied are supplemented with cuticular secretions (for review see Gorb, 2001). Secretion has been demonstrated in both alternative designs of the pads, hairy and smooth. A liquid film between two objects gives rise to adhesive forces because of surface tension and viscosity (Federle et al., 2002). Pad fluids have been reported from hairy adhesive pads of reduviid bugs (Edwards and Tarkanian, 1970), flies (Bauchhenss and Renner, 1977; Bauchhenss, 1979; Walker et al., 1985), coccinellid beetles (Ishii, 1987), and from the smooth pads of cockroaches (Roth and Willis, 1952), aphids (Lees and Hardie, 1988; Dixon et al., 1990), and bugs (Hasenfuss, 1977, 1978; Ghasi-Bayat and Hasenfuss, 1980). In the adhesive pads of insect legs, epidermal cells with secretory activity usually contain at least two types of vesicles: electron-lucent and electron-dense (Bauchhenss and Renner, 1977; Bauchhenss, 1979; Lees and Hardie, 1988). We have previously hypothesized that the function of such a complex mixture of the pad secretion (hydrophobic and hydrophilic; Gorb, 2001) might be attachment to a variety of different substrates. The literature provides lines of evidence for the biphasic nature of the fluid of adhesive pads of flies (Gorb, 2001; Langer et al., 2004) and ants (Federle et al., 2002). The most comprehensive comparative chemical analysis of attachment pad compounds and regular cuticle has been presented by Vötsch et al. (2002), who investigated the locust. On the basis of their results, the authors suggested that the fluid is a kind of a coupling agent, promoting and strengthening adhesion between otherwise incompatible materials by providing the proximity of contact (Vötsch et al., 2002). Therefore, the lack of smooth endoplasmic reticulum in *K. biedouwense* is surprising. The abundance of RER in close association with large golgi complexes rather suggests that proteins are secreted by the arolium gland cells. In addition, the more or less rounded, electron dense secretion vesicles of various sizes that were found within the gland cells are probably filled with proteinaceous material (especially in combination with abundant RER; Quennedey, 1998). Smooth endoplasmic reticulum, which is often found in defensive glands that produce nonproteinaceous secretions (Happ et al., 1966; Crossley and Waterhouse, 1969; Schumacher, 1971; Araujo and Pasteels, 1985), scent glands of Lepidoptera (Percy, 1974; Percy-Cunningham and MacDonald, 1987) and in glands secreting pheromones or lipids (Quennedey, 1972; Cruz-Landim et al., 2005), is apparently absent in the arolium gland cells of *K. biedouwense*. However, the chemical composition of the secretion remains to be investigated.

Function of the Arolium and Its Gland

A gland located directly in the arolium has never been described in detail before. In so-called arolium glands that were found in ants (Hölldobler and Palmer, 1989) or wasps (Billen, 1986) the glandular tissue is located within the tarsus and only the tarsal gland reservoir is connected to the lumen of the hollow arolium. The arolium gland is found in all three leg pairs and is a common gland in all Hymenoptera (Billen and Morgan, 1998; Tijskens et al., 2002; Billen et al., 2005; Billen, 2009). Most of these glands are considered to produce secretions for adhesion (Hölldobler and Palmer, 1989).

This study clearly shows that representatives of Mantophasmatodea studied utilize fluid in the contact zone. Our microscopic observations and high-speed video recordings show that in contrast to hymenopterans, a lateral expansion and increase in volume during attachment, or a decrease in volume when detaching from the surface (Baur and Gorb, 2001; Federle et al., 2001), does not occur in heelwalkers (see Fig. 9).

The ablation experiments provide evidence that Mantophasmatodea do not use their arolia for the perception of vibrational signals. All tested *K. biedouwense* males reacted with increased locomotor activity, searching behavior and drumming, when stimulated with the conspecific female call, irrespective of the absence or presence of arolia. The distal tarsi and arolia are therefore not involved in vibration reception; the scolopidial organ found within the 5th tarsomere is probably only used for proprioception.

Experiments about the ability of *K. biedouwense* males with and without arolia to catch prey (*Drosophila* sp.) also indicate that they do not need their arolia to catch prey. However, we cannot fully exclude that they may play a role when large prey is attacked.

Behavioral observations and results of our attachment experiments suggest that the attachment forces generated by euplantulae seem to be sufficient to resist gravity force caused by the body mass. Arolia are mainly needed for strong attachment on smooth surfaces and/or to secure attach-

ment during substrate disturbances or rapid wind pulses. *K. biedouwense* males were not able to move upside down on the glass plate when the arolia of all legs had been ablated. We therefore conclude that the function of the arolium is strong adhesion to the substrate, when moving on a smooth surface. Additionally, females carrying their mates or individuals manipulating large prey often secured their attachment on the smooth ceiling even when they did not move (Fig. 8A,B). This function of the arolium to support the adhesion provided by euplantulae in an extremely short period of time (about 18 ms) is definitely useful for wingless insects living in vegetation.

The structure of the smooth cuticle at the lateral distal margin of the arolium (surface with corrugations, fibrous structure of the cuticle) as it has been previously demonstrated for many smooth attachment structures (Beutel and Gorb, 2001, Gorb, 2001, 2008; Scholz et al., 2008) provides additional evidence that the arolium of heel-walkers is used for attachment.

The main functional background of the characteristic habit of keeping the arolium uplifted and off the substrate is probably to keep the sticky surface away from debris and surface asperities that could contaminate or even damage the surface. Slifer (1950) already reported damage of the smooth cuticle of arolia of locusts when they were forced to move on sandpaper. Keeping the adhesive contact area small may additionally help to save tarsal secretion (Federle et al., 2002; Vötsch et al., 2002; Federle and Endlein, 2004). Too strong adhesion would probably also negatively affect the walking performance, which requires fast and easy attachment and detachment. A similar attachment strategy is known for hymenopterans, which spread their arolia only when their claws fail to grasp the substrate (Snodgrass, 1956; Federle et al., 2001). Similarly, sarcophagid flies do not apply their pulvilli to the substrate, when they stay or walk on rough horizontal substrate (Gorb, unpublished data).

We conclude that the arolium of Mantophasmatodea is only used when additional adhesion force is required (e.g., windy conditions). For example, strong adhesion could be helpful as a protection mechanism against predators, similar to the behavior described in the beetle *Hemisphaerota cyanea* that uses its attachment structures as a defensive device against ants (Eisner and Aneshansley, 2000). Additionally, males use their arolia to attach onto the dorsal surface of the females, when attempting copulation.

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