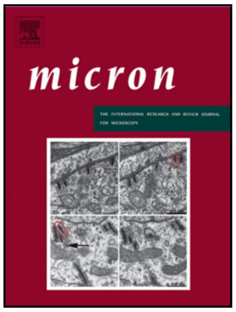

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Scanning Electron Microscopy of *Antarctophthirus microchir* (Phthiraptera: Anoplura: Echinophthiriidae): studying morphological adaptations to aquatic life

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Scanning Electron Microscopy of *Antarctophthirus microchir* (Phthiraptera: Anoplura: Echinophthiriidae): studying morphological adaptations to aquatic life

Abstract

The members of the Family Echinophthiriidae (Phthiraptera: Anoplura) are unique among insects because they infest hosts with an amphibious lifestyle. During their evolution they developed morphological traits that are reflected in unique features. The SEM is a helpful tool to analyze them. Knowing in detail the external structure of these lice is the first step to understand the whole process that derived from the co-adaptation of lice and pinnipeds to the marine environment. For the first time, we studied the external structure of all stages of an echinophthiriid louse. The results are discussed in the light of their evolutionary, functional, and ecological implications.

Highlights

Echinophthiriids are of the few insects that successfully adjusted to the marine environment lifestyle. The adaptation to the aquatic conditions is reflected in unique morphological features. We analyze for the first time all the stages of an echinophthiriid species under SEM. The results are discussed in the light of the evolutionary implications of these adaptations.

Keywords: *Antarctophthirus*, Echinophthiriidae, Anoplura, Phthiraptera, morphological adaptations, SEM images

1- Introduction

It is widely known that insects exhibit a great species diversity and enormous abundance. However, lice are the only insects that have become obligate and permanent parasites throughout their entire life cycles (Kim, 1985; Bush *et al.*, 2001). Exceptionally, a group of lice managed to advance further colonizing the marine environment (Kim *et al.*, 1975). The Echinophthiriidae is a family of sucking lice (Anoplura) that are specific to pinnipeds and the river otter. To cope with an amphibious lifestyle, pinnipeds have developed different insulation mechanisms (King, 1983) that are relevant to the biology of their lice.

Although most marine mammals rely on blubber for insulation, a layer of air trapped within the hair or fur serves as the primary insulator in fur seals and sea otters and keeps the skin dry when the animals are submerged (Yochem & Stewart, 2008).Therefore, lice infesting fur seals spend all their life cycle in a virtually “terrestrial” environment (Kim, 1975). However, true seals, walruses, and sea lions rely more on blubber for insulation, with a less dense pelage which becomes wet during immersions (Mostman Liwanag, 2008). Accordingly, echinophthiriids infesting these pinnipeds are exposed to truly aquatic conditions, and this requires specialized morphological and life-history adaptations for insects that were terrestrial in their origin.

As part of an ongoing project on the adaptations to marine lifestyle of the echinophthiriid *Antarctophthirus microchir* infesting South American sea lions (*Otaria flavescens*), we had the opportunity to study these lice under Scanning Electron Microscopy (SEM). SEM allows a detailed examination of specialized external structures to understand the morphological adaptations of this peculiar group of lice to such lifestyle.

Previous SEM studies of Echinophthiriidae were carried out in the following species: *Antarctophthirus trichechi* and *Echinophthirius horridus* (see Scherf, 1963); *E. horridus*, *Antarctophthirus callorhini* and *Proechinophthirus fluctus* (see Miller, 1971); *Antarctophthirus callorhini* and *Proechinophthirus fluctus* (see Kim, 1971); *Proechinophthirus zumpti* (see Castro *et al.*, 2002); *Antarctophthiru ogmorhini* (see Mehlhorn *et al.*, 2002), and *Lepidophthirus macrorhini* (see Green & Turner, 2004). Kim (1971) described all the developmental stages of the species infesting the Northern fur seal, *Callorhinus ursinus*, under light microscopy. This author analyzed the main morphological features of these lice and discussed them in relation to both the significance in the adaptation to the marine habitat and the microhabitats used by each species. However, the remaining publications neither include all life stages in the SEM analysis nor emphasized the particular features of echinophthiriids related to their aquatic lifestyle. Scherf (1963) and Miller (1971) focused on the sensorial structures of legs and antennas respectively. Mehlhorn *et al.* (2002) recognized the importance of scales, postulating a potential function as plastron, as it was previously proposed by Hinton (1976). Castro *et al.* (2002) used SEM as a tool to recognize several diagnostic characters. Finally, Green and Turner

(2004) discussed morphological specializations for the attachment of the louse onto the host. In the present study, we analyze under SEM all the stages of *A. microchir* from South American sea lions, identifying specialized and specific structures which are discussed in the light of their ecological, functional and evolutionary implications. This constitutes the first step to understand the adaptations of an originally terrestrial louse to the marine realm.

2- Materials and methods

2.1- Specimens examined

Lice were collected from sea lion pups in Punta León rookery (43°03'S, 64°30'W) in Patagonia, Argentina, during the 2006/07 breeding season. Pups were captured with a noose pole and restrained by two people, a third person collected the lice from the belly using a fine-tooth comb commonly used for treating human pediculosis, and lice were fixed in 96% ethanol. The mean manipulation time was 4'15" ± 37". Manipulation included measuring, weighing, tagging and combing the pup. Combing took about half of the whole manipulation time and stopped when no more lice were collected. Once pups were released their mothers readily accepted and nursed them, and all manipulated pups survived the study period. Lice were classified into nymphal stages and male and female adults following Leonardi *et al.* (2009). Ten adult males, ten adult females, ten 1st instar nymphs (N1), ten 2nd instar nymphs (N2), and ten 3rd instar nymphs (N3) of *A. microchir* were examined using SEM.

2.2- Scanning Electron Microscopy (SEM)

Specimens for SEM (ten of each life stage: five in dorsal view and five in ventral view, and two eggs) were dehydrated in an ethanol series, critical point dried in liquid CO_2, mounted on specimen stubs with conductive carbon paint, sputter coated with gold-palladium to a thickness of 25-30 nm in a Bio Rad-Sc 500 coating unit, and examined in a S-4100 Scanning Electron Microscope at 5 kV (Servei Central de Suport a la Investigació Experimental, Universidad de Valencia, Spain).

Denomination of morphological characters follows the criteria used by Leonardi *et al.* (2009): spines are pointed and spiral shaped setae, scales are flattened setae, and hairs are the long and thin setae.

3- Results

a- Description of life stages

A detailed description of adults and developmental stages was given in Leonardi *et al.* (2009).

Egg (Fig. 1) -Cemented individually to a single hair, is smooth, with an operculum distinctly raised, tapering to a blunt apex, without ornamentations. The operculum pointing toward the tip of the capillary fibre.

Nymphs 1 (Fig. 2) - Characterized by the absences of scales. Legs are proportionally larger in relation with the body size if they are compared with the legs of nymphs 2 and 3. The pattern of spines distribution is clearly visible: they are mainly and uniformly distributed along the abdominal segments.

Nymphs 2 (Fig. 3) - Scales appear in this stage, following the same pattern than in adults. As the ontogeny advances, hairs and scales become larger and denser, respectively. This is more notorious in the life stage nymphs 3.

Nymphs 3 (Fig. 4)– The pattern of chaetotaxys and main features are similar to adults. The scales and spines are denser than in nymph 2. Occipital apophyses of thorax converge at apex.

Adults (Fig. 5) -The absence of thoracic and sternal plates is noticeable, with the whole body mainly covered by scales. Females (Fig. 5a) are larger and their abdomens more rounded than in males (Fig. 5b); the genital opening of females is surrounded by a fringe of setae.

b- Main structures

Chaetotaxy - One of the main features of echinophthiriids is the presence of modified setae in three types: spines (Fig. 6a), scales (Fig. 6b) and hairs (Fig. 6c). The scales of *A. microchir* cover the whole abdomen and are disposed in an imbricate pattern (Fig. 7).

Spiracles (Fig. 8) – The figure show the disposition of spiracles. Note that scales are not particularly disposed surrounding them. The spiracles are modified as a membranous structure, which is clearly visible in figure 8c.

Legs (Fig. 9) –The forelegs are smaller and slender than middle and hind legs (Fig. 9a). The tibia-tarsi of the second and third pair of legs of *A. microchir* are modified to hold to the hairs of the host (Fig. 9b), while the first are smaller and weaker (Fig. 9c). The distotibial process (Fig. 10) present in the modified legs of *A. microchir* is characterized by the presence of clam-shaped setae (Fig. 10b).

Head (Fig. 11) - The head has no eyes (previous figures of each stage); a haustellum (Fig. 11a) is present with 8 large hooks disposed in 2 rows (Fig. 11b), without any other processes.

Antennae (Fig. 12) – Antennae with 5 segments; the basal segment with a spine, already present in the first nymphal stage. The terminal segment with sensillas, 2 pore organs, sensila basiconica not notorious (Fig. 12b).

Thorax- The thorax is trapezoidal, stronger in the middle and hind legs. Occipital apophyses converge to the apex from nymph 1 to nymph 3.

Abdomen- The abdomen is membranous and with a squamous texture. It is rounded and pointed in males. Except for nymphs 1, the abdomen is covered with scales in all stages.

4-Discussion

For the first time all developmental stages, including eggs, of an echinophthiriid species are analyzed under scanning electron microscopy (SEM) in the framework of morphological adaptations to the marine lifestyle. As mentioned above, echinophthiriids are of the few insects that successfully adjusted to the marine environment. During their evolution they developed morphological traits that are reflected in unique features. The SEM is a helpful tool to analyze them. Knowing in detail the external structure of these lice is the first step to understand the whole process that derived from the co-adaptation of lice and pinnipeds to the marine environment.

The egg is cemented individually to a single hair, unlike *Proechinophthirus zumpti* that places its eggs both individually or forming clusters (Castro *et al*., 2002). Mehlhorn *et al.* (2002) observed the same pattern described herewithin *A. ogmorhini* from Weddell seals. While the operculum of both *A. microchir* and *A. ogmorhini* (see Mehlhorn *et al*., 2002) lack ornamentations, the operculum of *P. zumpti* is irregular and little elevated (Castro *et al.*, 2002). The relative position of the eggs in relation with the capillary fibre suggests that the oviposition behaviour of *A. microchir* is similar to that of other species of the genus, as *A. ogmorhini* (Mehlhorn *et al*., 2002). It is generally accepted that egg ornamentations are involved in the hatching mechanism (Berman *et al*., 1980). Therefore, despite being a simple structure, the egg morphology and the oviposition pattern could be a taxonomic and a behavioural feature, respectively, characteristic at generic level.

As noted by Leonardi *et al.* (2009) the nymph 1 is characterised by the absence of scales, which are present in all other instars. Nymphs 2 and 3 are very similar, differing in the abundance and density of abdominal scales and in the occipital apophyses (Leonardi *et* *al.,* 2009). This pattern was previously reported by Kim (1971) and seems to be characteristic of the family.

The presence of setae in Anoplura is primarily related to a sensitive function (Kim and Ludwig, 1978). One of the main features of echinophthiriids is the presence of modified setae (Kim, 1971; Leonardi *et al*., 2009) in three types: spines, scales and hairs. Furthermore, this unique pattern of chaetotaxyis the most distinctive morphological feature of echinophthiriids and, therefore, this might suggest that it is closely related to their marine lifestyle (Kim 1971, 1985). However, the precise function of these morphological adaptations is still under discussion.

Mehlhorn *et al*. (2002) observed that the length of spines may be species-specific. The spines of *A. microchir* seem to be more similar to those of *Echinophthirius horridus*, *Lepidophthirus macrorhini* and *A. trichechi* (Murray 1976; Scherf 1963), while the spines of *A. ogmorhini* are shorter (Mehlhorn *et al*., 2002). The distribution pattern of spines is conservative through the ontogeny from nymph 1 to adults. This fact, considering the distribution, size and shape of spines would suggest a sensitive function. The observations of Nuttal (1918), who reported the existence of nervous associated to spines, support this hypothesis.

Another possible function of spines is related with protection against low temperatures. Mehlhorn *et al*. (2002) established that spines of *A. ogmorhini* play a role retaining seal sebum as a protection against low temperature when the hosts, Weddell seals, reproduce in Antarctica. However, spines are also present in *A. microchir* parasitizing the South American sea lions, which breed during the austral summer when ambient temperatures may raise up to 40º C, although these sea lions also swim in waters at about 8–9º C. Therefore, the function of spines retaining the waterproof sebum of the host could be related to temperature. Moreover, pinnipeds are a monophyletic group with origins dating to at least the late Oligocene (27 MYA) and with a hypothesized North Pacific origin (Berta, 2009), implying that ancestral pinnipeds had to dealt with extremely cold conditions (Bowen, 2007).

The presence of scales is characteristic of the genus *Antarctophthirus* (Kim, 1971), with several authors having discussed their function. Mehlhorn *et al*. (2002) established that they are typical of Antarctic and Arctic lice. However, species of *Antarctophthirus* are distributed worldwide (Kim, 1971). Hinton (1976) proposed that scales work as a plastron, i.e. a surface to retain a gas film, creating a water-air interface. The scales of *A. microchir* cover the whole abdomen and are disposed in an imbricate pattern; hence, this arrangement of scales could allow the retention of air among them. Murray (1976) argued that the scales cannot be related to aquatic respiration because there is no connection with the tracheal system. On the contrary, as it was explained by Hinton (1976), a plastron involves cutaneous respiration. Scales are not particularly disposed surrounding the spiracles; this would indicate that aquatic respiration is independent of the principal tracheal system. Echinophthiriid lice lack sclerotized structures in the abdomen, which would provide a surface for gas exchange (Kim, 1971). A hypothesis to test would be to find setal tracheal gills as observed in diving beetles (Kehl & Dettner, 2009). In this group of these coleopterans, the gas exchange is through tracheated setae, which act as tracheal gills (Kehl & Dettner, 2009).

The hypothesis that scales are related to respiration is also associated with ecological and physiological hosts' features. Differences in the presence of scales among echinophthiriid lice are related to the habitat inhabited by each species. Fur seals have developed a dense long-haired fur that creates a layer of trapped air when the animal is submerged. This insulation mechanism generates a virtually 'terrestrial' environment for species of *Proechinophthirus*, e.g. *Proechinophthirus fluctus* on Northern fur seals, *Callorhinus ursinus* (Kim, 1975). Consequently, these species are devoid of scales. In the case of species of *Antarctophthirus*, they are exposed to aquatic conditions because the host pelage becomes wet during immersions (Mostman Liwanag, 2008). Therefore, *Antarctophthirus* lice may need these specialized structures to breathe and survive underwater. Nymphs 1 of *A. microchir* lacks of specialized abdominal scales that should assist more developed instars in obtaining oxygen underwater. In fact, Aznar *et al*. (2009) suggested that nymphs 1 might be affected negatively by first immersions because their abundance decreased in pups that started to swim. In other words, the decrease of nymphs 1 when pups become wet would be a consequence of the absence of scales and, therefore, the impossibility to form and maintain an air blanket underwater. Moreover, the absence of scales on nymphs 1 could be interpreted as the retention of a primitive morphological feature, which is also are present in *Proechinophthirus* species (Kim 1971, 1988).

According to Green & Turner (2004), the lack of rigid plates may allow the compression of the body under high pressure, avoiding damage. Therefore, the scales could be involved in the protection of the cuticle against low temperatures, as was originally proposed by Hinton (1976).

Hairs probably have a sensorial function and are also useful as taxonomic characters because they are species specific (Mehlhorn *et al*., 2002), i.e. *A. ogmorhini* has groups of four hairs (Mehlhorn *et al*., 2002), *A. trichechi* has two on each side (Scherf 1963) and *A. microchir* has long thoracic hairs (Leonardi *et al*., 2009).

An important adaptation of echinophthiriids is reflected in the morphology of their legs. As a consequence of their marine lifestyle, echinophthiriids are blind (Kim, 1985) and present their first pair of legs modified to have a sensitive function. The tibia-tarsi of the second and third pair of legs of *A. microchir* are modified to hold to the hair of the host. At the moment, there is no available information about a potential relation between the size of the leg and the diameter of the hair of the host. However, Reed *et al*. (2000) found a positive correlation between the diameter of the hair of gophers (family Geomyidae) and the rostral groove dimensions of their chewing lice of the genus, *Geomydoecus*. Chewing lice use the rostral groove to grasp to a host hair pelage while sucking lice use their legs. Therefore, it is possible that a correlation might exist between leg dimensions and host hair diameters among pinnipeds and their lice, and/or the shape of the clam-shape setae and the ultrastructure of the hair fibre. Further research is needed to prove its significance.

As noted above, adaptations of pinnipeds' lice to the marine environment are reflected in many unique morphological features. These adaptations will be better explained and understood by additional information on biology, ecology and physiology of *A. microchir*. Besides the morphological adaptation of echinophthiriids, the present work has a taxonomic value. It has been proposed that *A. microchir* from different sea lion hosts represents a complex of cryptic species (Leonardi *et al*., 2009). The morphological information and the description of the ultrastructure reported in the present work constitute a valuable baseline to compare *A. microchir* from different host species and to finally resolve the taxonomic question of this complex of species.

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Figure captions

Figure 1- SEM photomicrographs of the egg of *Antarctophthirus microchir*, individually cemented to a single hair. (a) Lateral view of the egg (E) attached to a sea lion hair (H); notice the cement (C) with which is fixed and the operculum (O), raised and without ornamentations; (b) View from the operculum, note the smooth surface; (c) Detail of the operculum.

Figure 2- SEM photomicrographs of the first instar of *Antarctophthirus microchir*, showing absence of scales. (a) The whole N1 (Bar = 250 μm), characterized by the absence of scales in abdomen (Ab) nor thorax (T); note that legs (L1, L2 and L3 fore, middle and hind legs, respectively) are significantly bigger in proportion to the body; the antenna is four segmented; (b) Ventral view of a the same stage, notice the membranous structure of the body surface and the notorious haustellum (Ha) (Bar = 100 μm); (c) Detail of the abdomen, where is clearly visible the lack of scales (Bar = 25 μm).

Figure 3- SEM photomicrographs of the second instar of *Antarctophthirus microchir*. (a) Dorsal view, notice the presence of abdominal scales; (b) Ventral view. Bar= 250 µm. References: An, antenna; Hd, head; T, thorax; Ab, abdomen, L1-L2-L3 respectively, fore, middle and hind legs.

Figure 4- SEM photomicrographs of the third instar of *Antarctophthirus microchir*. (a) Dorsal view; (b) ventral view. Notice the similarity between the third developmental stage and adults. Bar= 500 µm. References: An, antenna; Hd, head; T, thorax; Ab, abdomen, L1-L2-L3 respectively, fore, middle and hind legs.

Figure 5- SEM photomicrographs of adults of *Antarctophthirus microchir*. (a) Ventral view of female, in which is clearly visible the genital opening (GO) surrounded by a fringe of setae, the abdomen is more rounded than in males; (b) Ventral view of male, note the development of the middle and hind legs, with the distotibial process (DTbP) and claws (Cw) used to hold the hair of the host. Bar= 500 µm.

Figure 6- SEM of modified setae of *Antarctophthirus microchir*. (a) General view of spines (Spn), scales (Sc) and hairs (Hrs) (Bar= 50 µm); (b) spines, with their typical grooves (Bar= 10 µm); (c) scale (Bar= 10 µm); (d) hairs (Bar= 50 µm).

Figure 7- SEM photomicrographs of the plastron of *Antarctophthirus microchir.* (a) Arrangement of scales in an imbricate pattern (Bar= 50 µm); (b) The disposition of the scales might form a plastron (Bar= 25 µm).

Figure 8- SEM photomicrographs of the spiracles (Sp) of *Antarctophthirus microchir*. (a) General view of the three lateral spiracles (Bar= 100 µm); (b) Detail of a spiracle (Bar= 25 µm); (c) Detail of the membranous closure system.

Figure 9- SEM photomicrographs of the ventral view of a female of *Antarctophthirus microchir* which is attached to a hair of the host (H) by means of the claws (Cw) of the middle and hind legs. (a) General view (Bar= 500 µm); (b) detail of the first leg, which is modified to a sensitive function (Bar= 100 µm); (c) Detail of the third leg, notice the tibia-tarsi strongly modified for grasping, the distotibial process (DTbP) is highly developed and the claw is strong (Bar= 100 µm).

Figure 10- SEM photomicrographs of the legs modified as holdfast structures. (a) General view of the tibia- tarsi segment. References: Cw, claw; DTbP, distotibial process; CShS, clam shaped seta; (b) Detail of the clam shaped setae, probably with taxonomic value.

Figure 11- SEM photomicrographs of the head of a nymph of *Antarctophthirus microchir*. (a) View of the haustellum (Ha), (b) Detail of the hooks used to perforate the skin of the host.

Figure 12- SEM photomicrographs of the antenna of *Antarctophthirus microchir*. (a) See the five segments of the antenna; (b) Detail of the terminal segment with the sensorial at the extreme.

Figure1

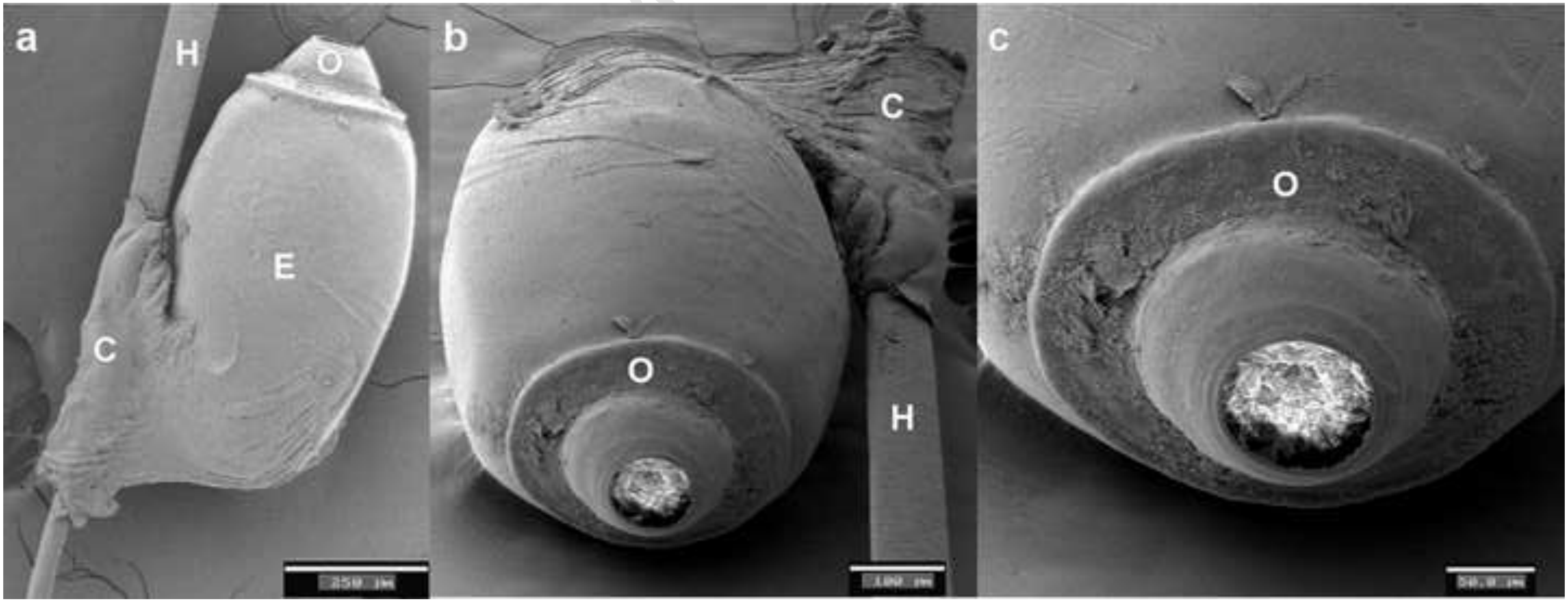

Figure2

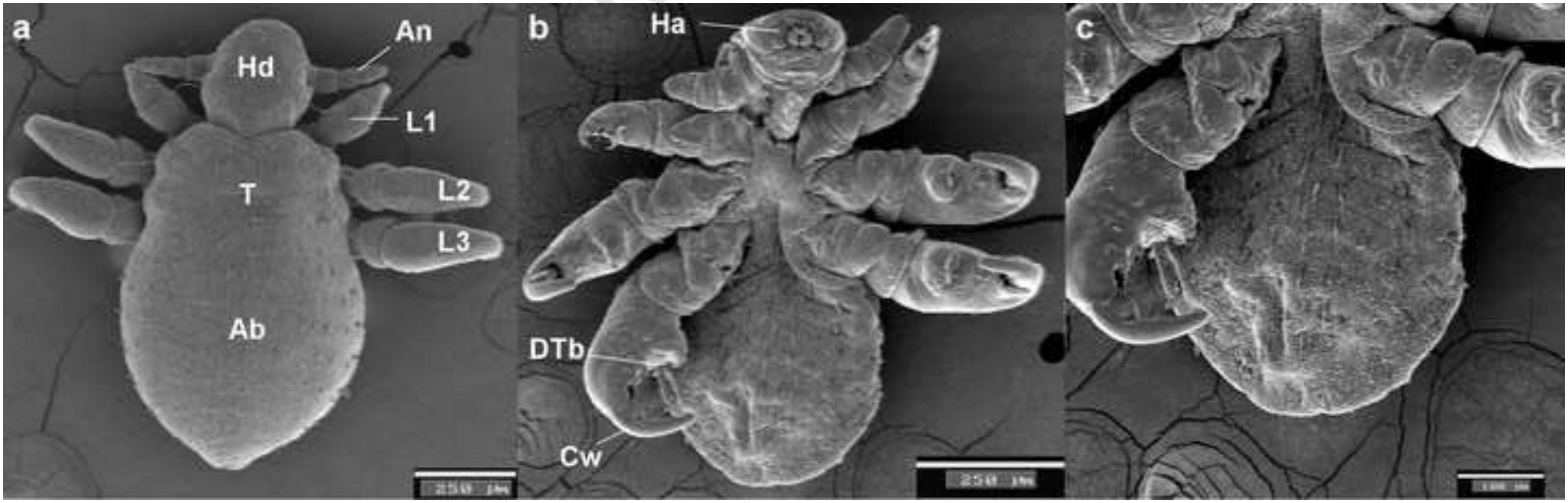

Figure3

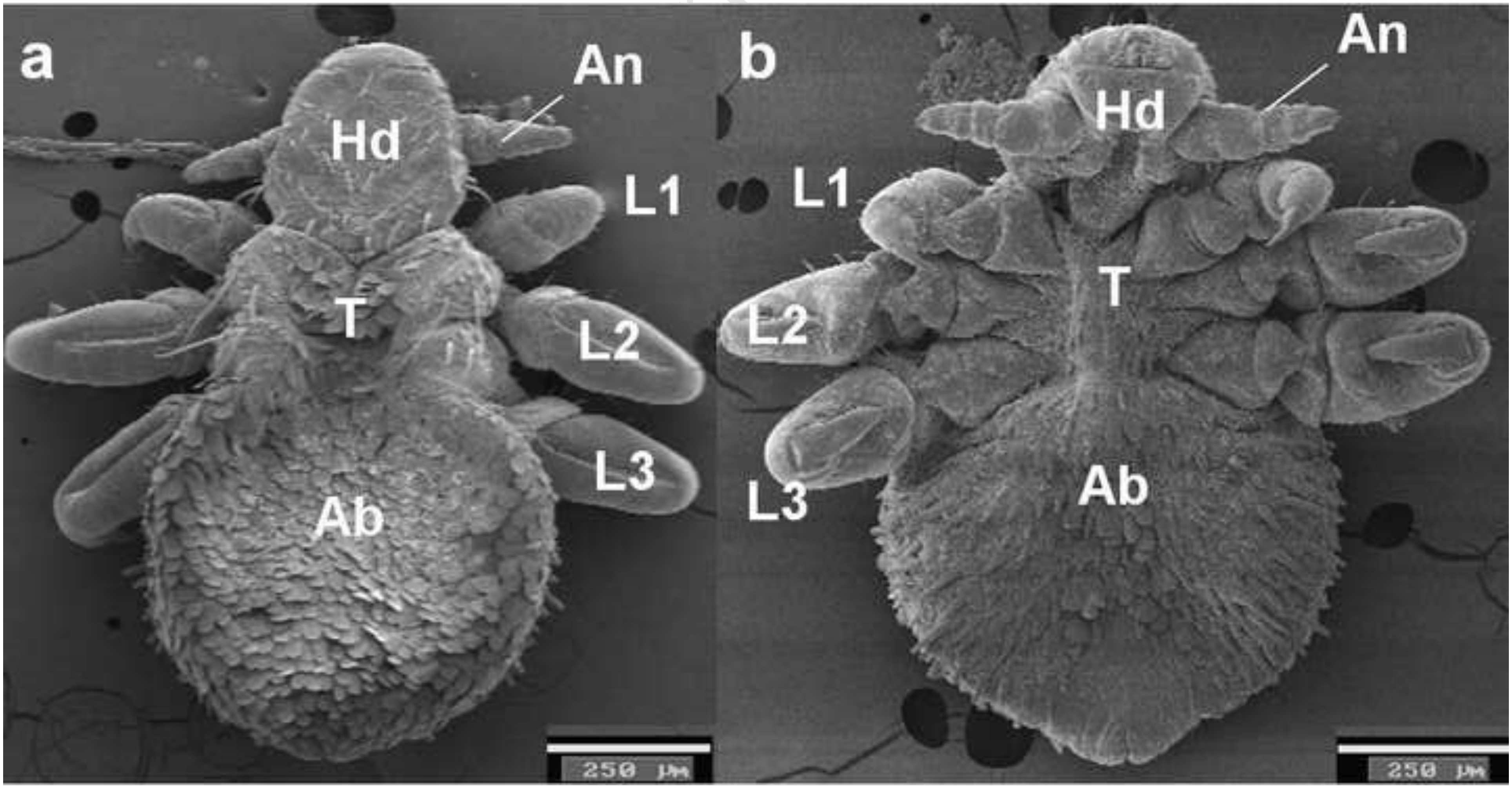

Figure4

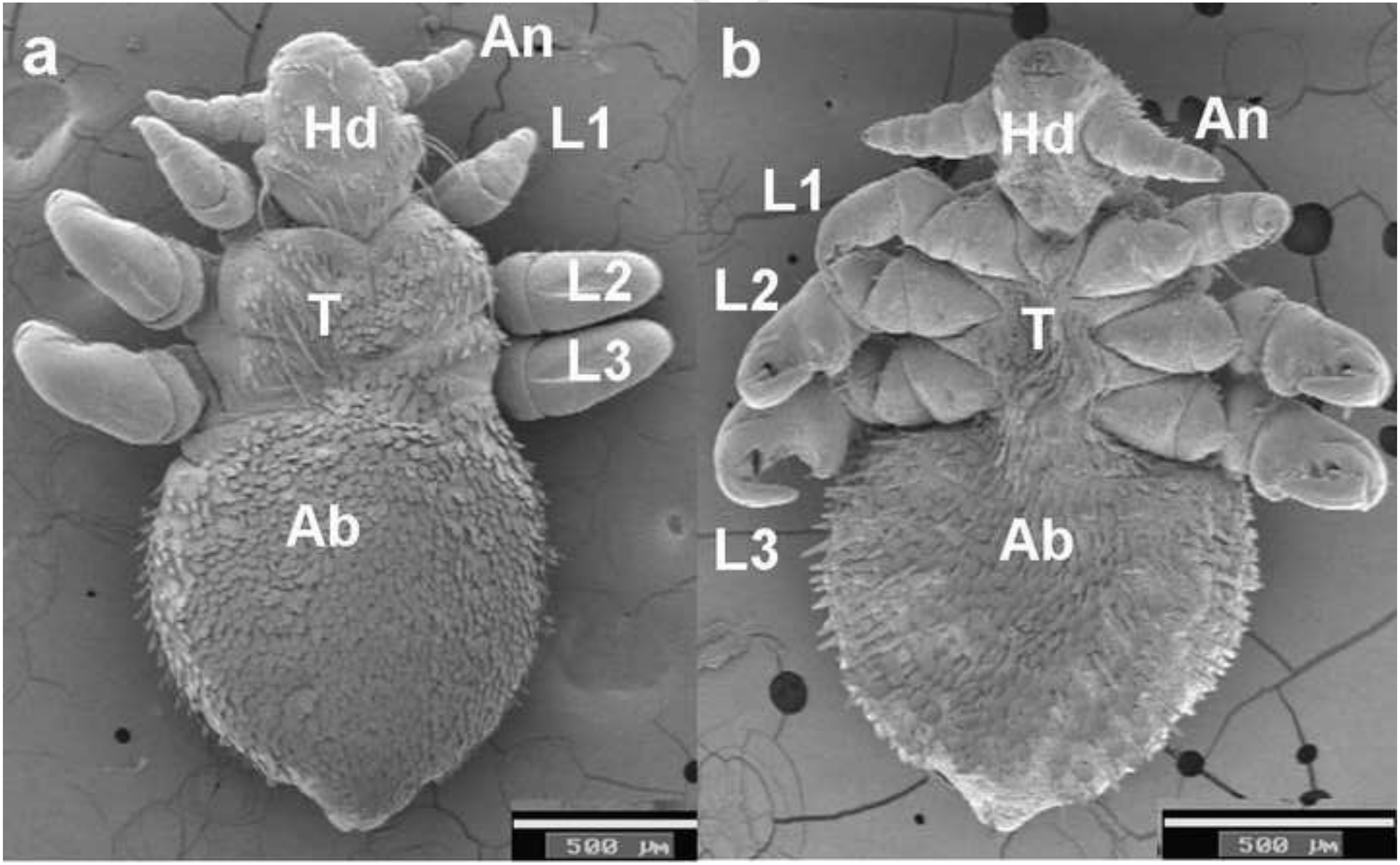

Figure5

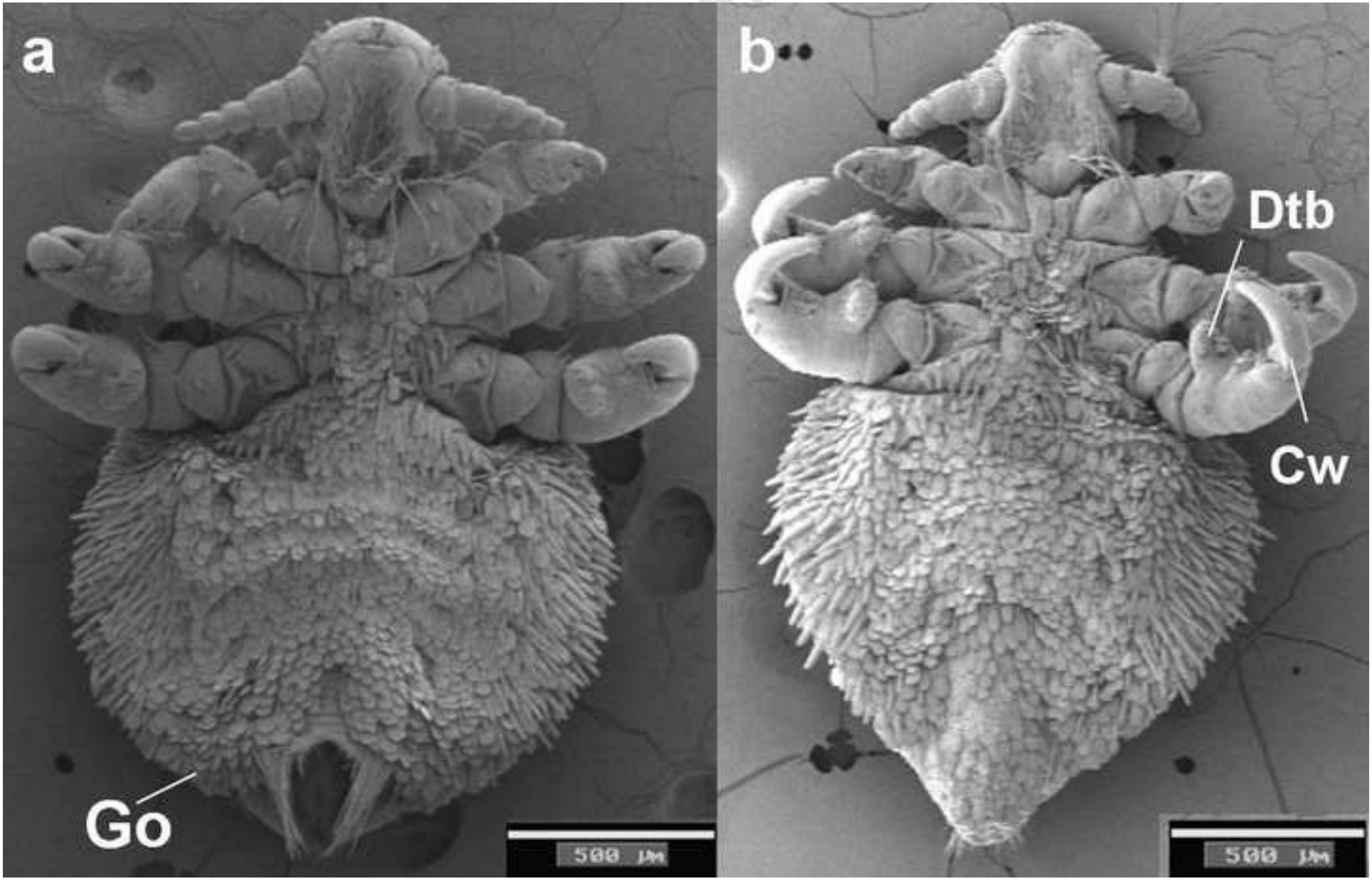

Figure6

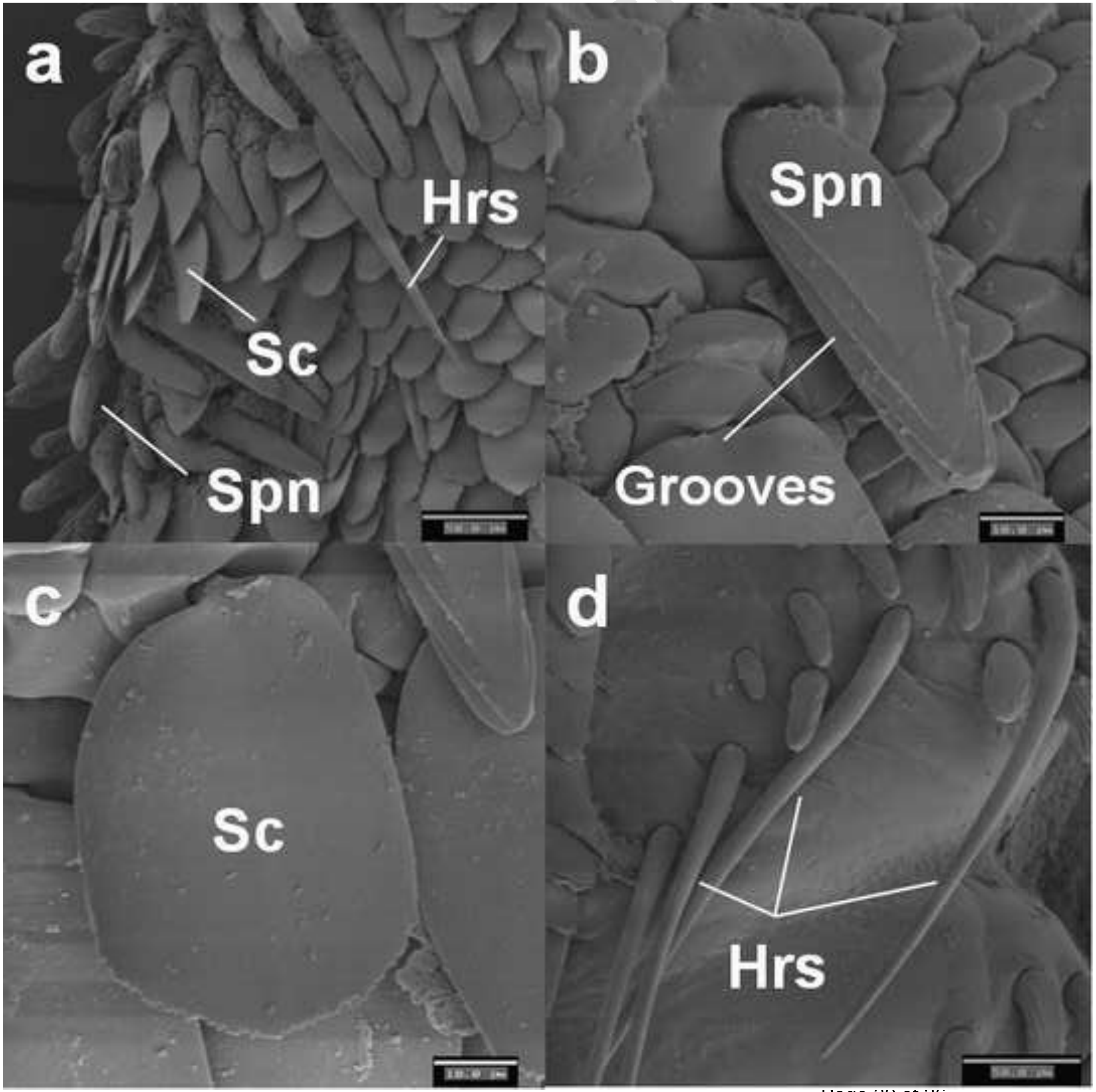

Figure7

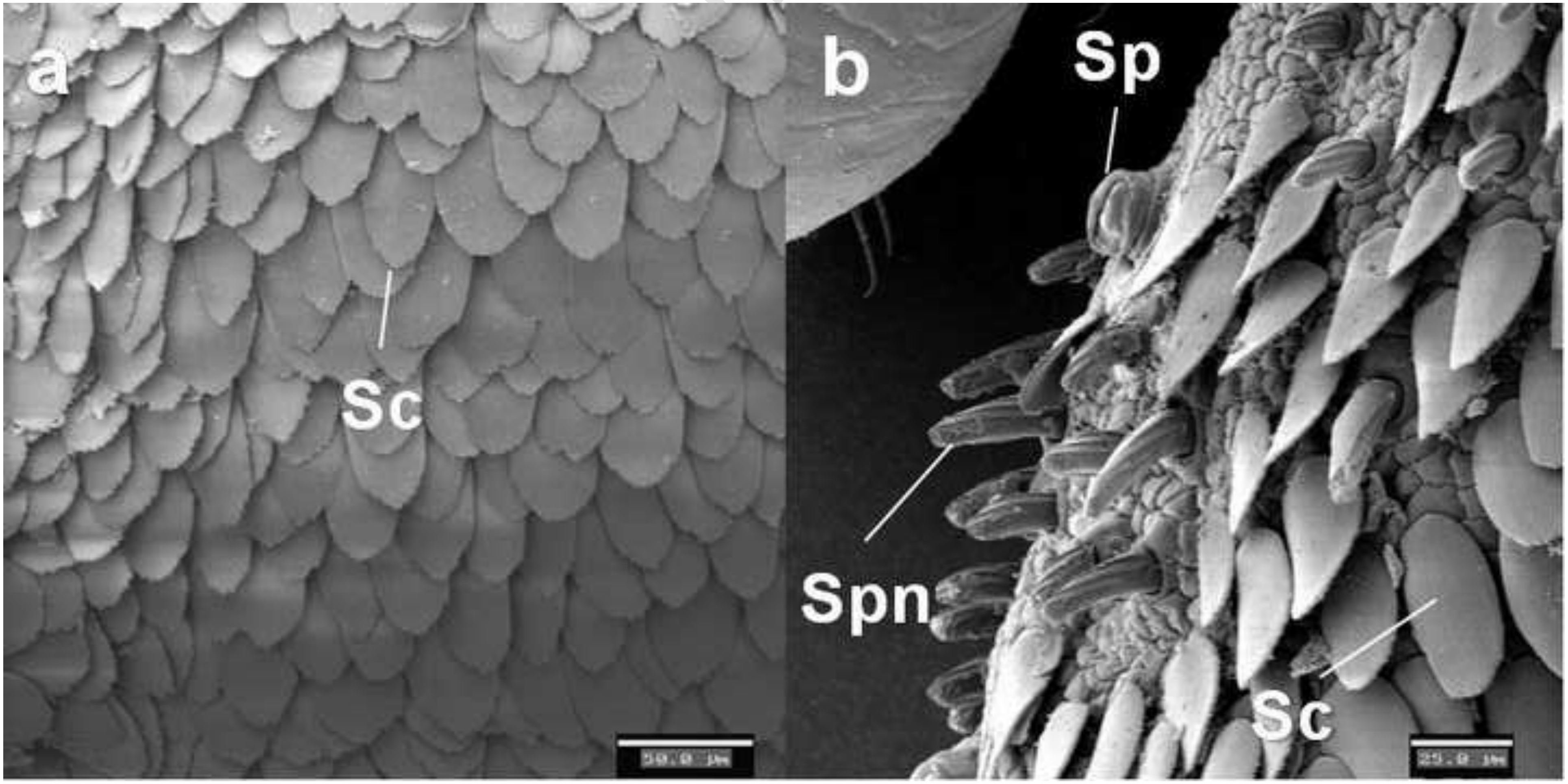

Figure8

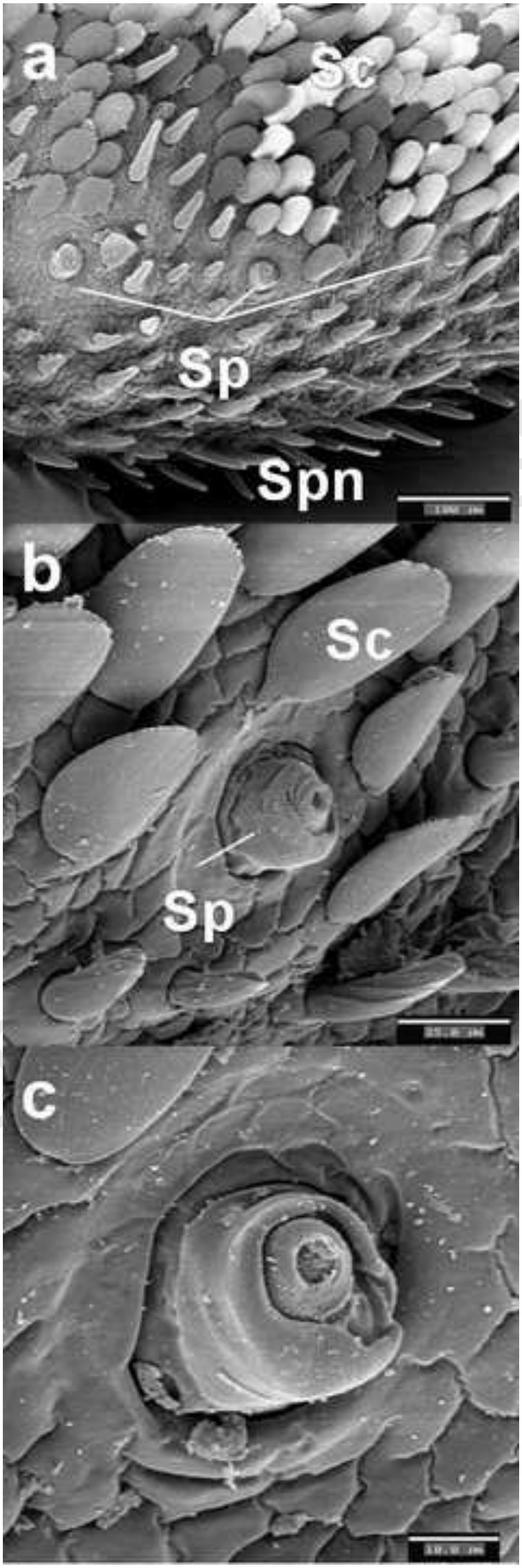

Figure 9

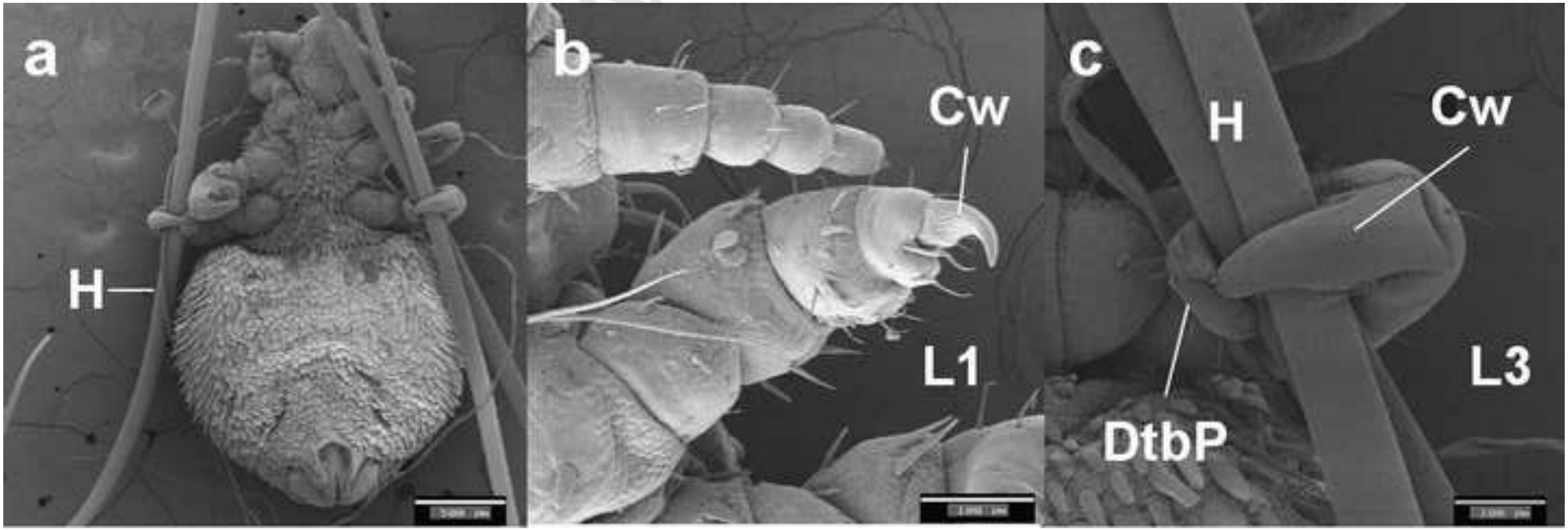

Figure 10

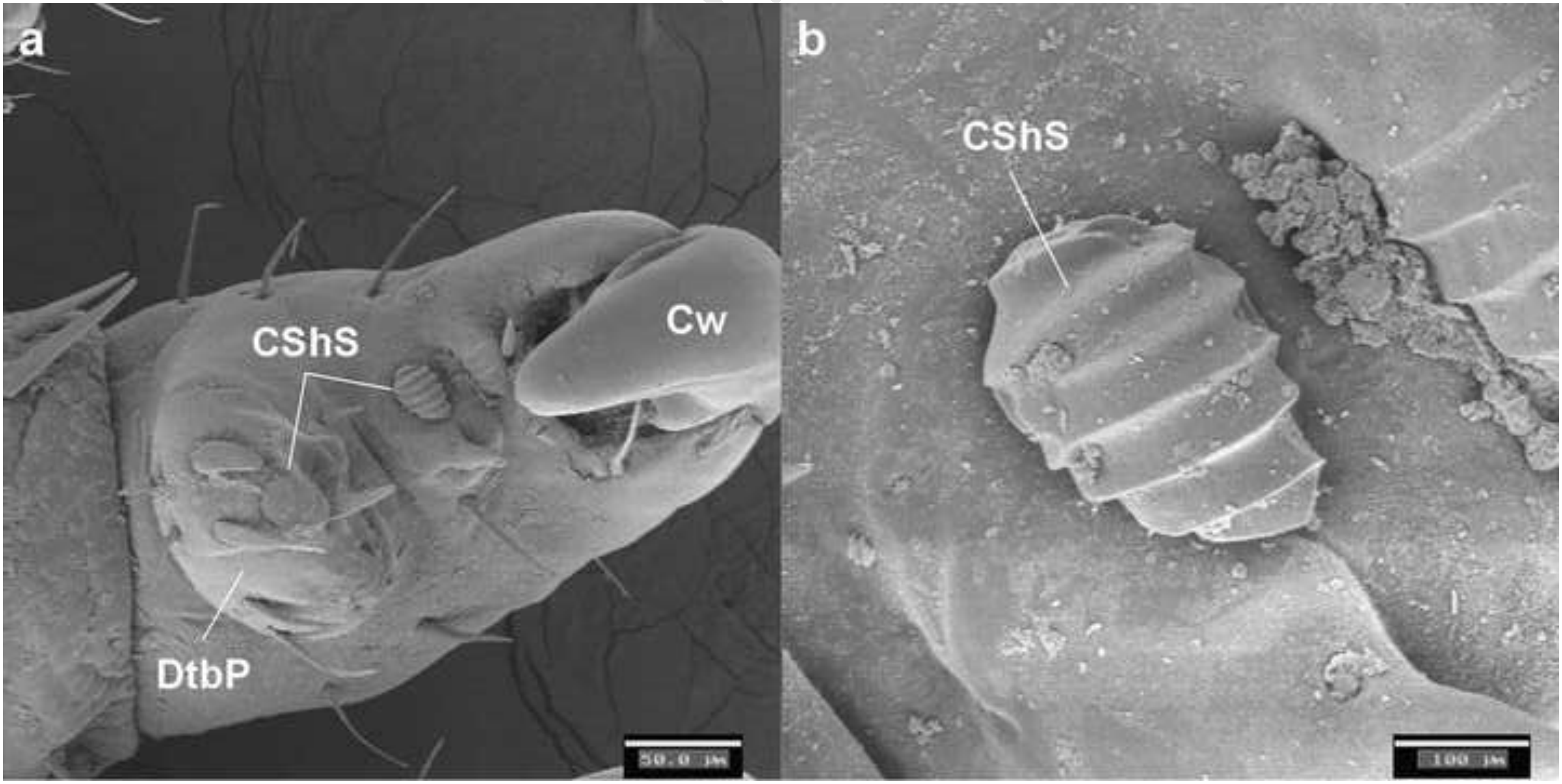

Figure 11

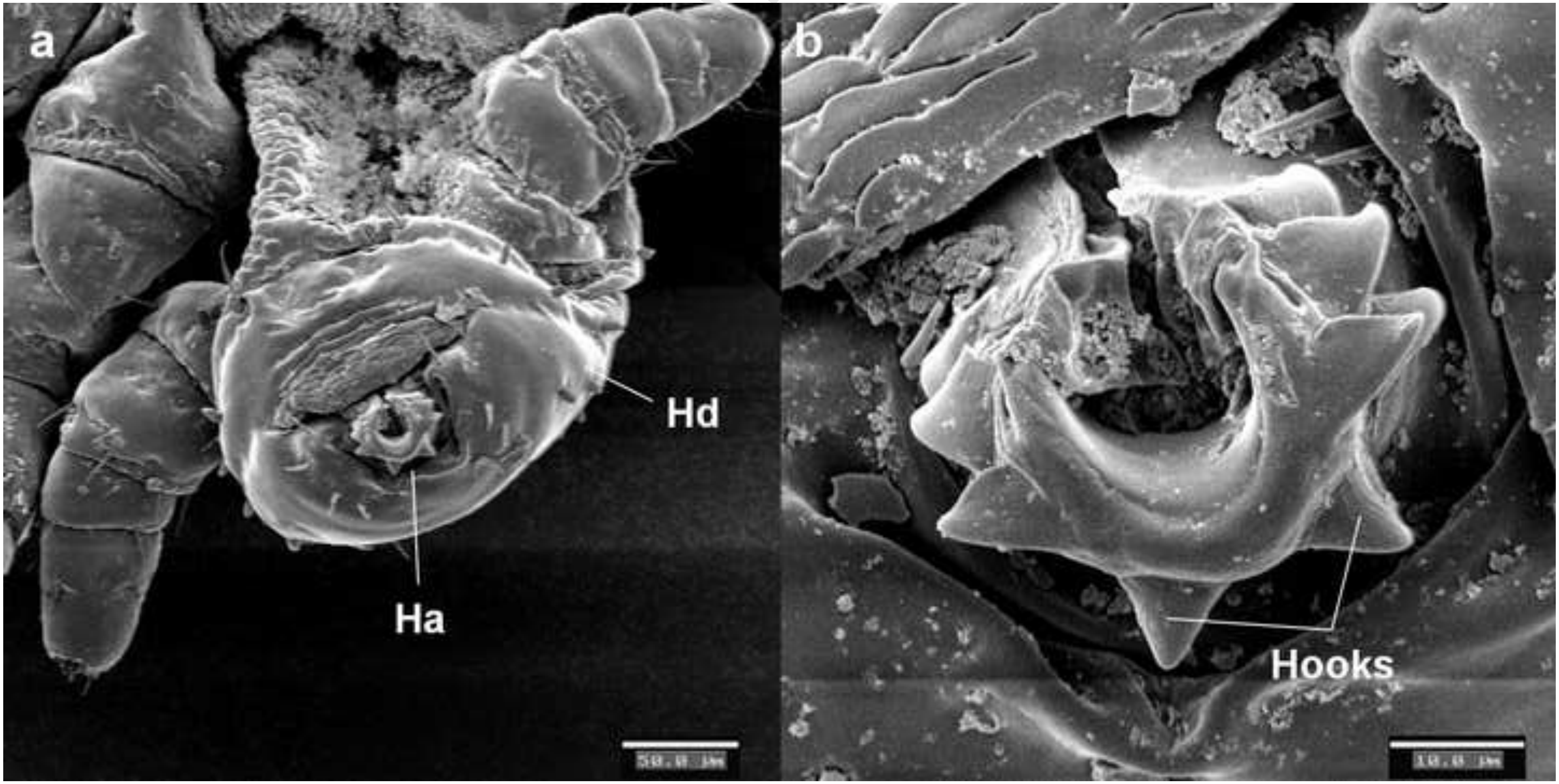

Figure 12