

Ultrastructural Comparison of External Morphology of Immature Stages of *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (Nyssomyia) whitmani* (Diptera: Psychodidae), Vectors of Cutaneous Leishmaniasis, by Scanning Electron Microscopy

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Ultrastructural Comparison of External Morphology of Immature Stages of *Lutzomyia* (*Nyssomyia*) *intermedia* and *Lutzomyia* (*Nyssomyia*) *whitmani* (Diptera: Psychodidae), Vectors of Cutaneous Leishmaniasis, by Scanning Electron Microscopy

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ABSTRACT *Lutzomyia* (*Nyssomyia*) *intermedia* (Lutz & Neiva 1912) and *Lutzomyia* (*Nyssomyia*) *whitmani* (Antunes & Coutinho 1939) (Diptera: Psychodidae) are vectors of American cutaneous leishmaniasis in several endemic regions of Brazil. We analyzed the external morphological aspects of the immature stages of these two vectors by using scanning electron microscopy. In general, the larval stages of the two species are morphologically similar, although some differences were noted. Detailed examination of the eggs of both species revealed similar exchorionic ornamentations of unconnected parallel ridges. The larval head capsules are well defined, heavily sclerotized, and bear prominent chewing mouthparts. The abdominal segments are easily recognized by the presence of prolegs on their ventral surfaces. The morphology of the anal lobe on the terminal abdominal segment differs between the two species. We found the following three types of sensillae inserted on the antennae: 1) clavate basiconic; 2) small, blunt coeloconic; and 3) multiporous clavate coleoconic. In addition; five subtypes of trichoid sensillae were found on the larval body: 1) long, 2) short, 3) curved long, 4) brush-like, and 5) weakly brush-like. The caudal filaments located on the last abdominal segment were recognized as long trichoid sensillae. We observed pores on the surface of the clavate coelonic sensillae and on the caudal filaments that presumably function as chemoreceptors. The larvae of the two species show similarities in the lobular-form antennae of L1 larvae, which changes to digitiform in second instar (L2), L3, and L4. This study demonstrated that the external surface of the eggs and larvae of *Lu. intermedia* and *Lu. whitmani* are morphologically similar, but they can be distinguished by details in the microanatomy observed by scanning electron microscopy.

KEY WORDS *Lutzomyi. intermedia*, *Lutzomyi whitmani*, immature stages, scanning electron microscopy

Lutzomyia (*Nyssomyia*) *intermedia* (Lutz & Neiva 1912 and *Lutzomyia* (*Nyssomyia*) *whitmani* (Antunes & Coutinho 1939) are South American phlebotomine sand flies and proven vectors of *Leishmania braziliensis* (Vianna 1911), the causative agent of American cutaneous leishmaniasis in Brazil and other South American countries (Vexenat et al. 1986; Jones et al. 1987; Rangel et al. 1990; Hashiguchi et al. 1992; Salomón et al. 1995, 2001a, b; Brito et al. 2002; Souza

et al. 2002). These two closely related species (Mazzone et al. 2002) are sympatric in some endemic areas (Oliveira dos Santos et al. 1993, Miranda et al. 2002).

Sand flies are holometabolous insects with four life cycle developmental stages: egg, larva, pupa, and adult. Pioneering taxonomic studies of immature stages of phlebotomines used optical microscopy as a common tool (Barreto 1941; Guitton and Sherlock 1969; Ward 1972). Later, Ward and Ready (1975) introduced scanning electron microscopy (SEM) to describe details of the exchorionic ornamentation of eggs of 13 species of Brazilian phlebotomines. Thereafter, other studies used SEM to reveal ultrastructural details of various immature stages of *Phlebotomus tobbi* (Adler & Theodor, 1930) (Killick-Kendrick et al. 1989), and pupae and first and fourth (L1 and L4) instars of *Lutzomyia longipalpis* (Lutz & Neiva 1912) (Leite et al. 1991; Leite and William 1996, 1997). Secundino and

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Pimenta (1999) described morphological characteristics of all developmental stages of *Lu. longipalpis* through SEM. Until now, only optical microscopic observations have been made on the external morphology of *Lu. intermedia* and *Lu. whitmani* (Barreto 1941). This is the first time SEM has been used to compare ultrastructural features on the external surfaces of immature stages of these two sympatric vector sand flies.

Materials and Methods

Sand Flies. Sand flies were captured live with CDC light traps (Sudia and Chamberlain 1962) at Corte de Pedra (13° 32' S, 39° 25' W) in the State of Bahia, Brazil, and transferred to a colony cage (30 cm³) where they were blood fed on hamsters and provided a 50% sugar solution ad libitum. After blood feeding, the flies were held in the colony cages at 26°C and 60% RH to allow for mating and digestion of the bloodmeal. Three or 4 d postfeeding, the bloodfed females were transferred to oviposition containers (Modi and Tesh 1983), where they laid their eggs. Upon hatching, the larvae were fed the appropriate diet (Secundino and Pimenta 1999) to complete their life cycle. Twenty specimens of each developmental stage, except pupa, were collected (eggs and the four instars of larvae), washed in phosphate-buffered saline, and immediately fixed overnight in 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, at room temperature.

Scanning Electron Microscopy. Fixed samples were processed for SEM as described previously (Secundino and Pimenta 1999). Briefly, samples were post-fixed in 1% OsO₄ plus 0.8% potassium ferricyanide in 0.1 M cacodylate buffer, pH 7.2, and dehydrated in ethanol at increasing concentrations of 50, 70, 90, and 100%. After that, the samples were critical point-dried under CO₂. They were then coated with a 20-nm layer of gold particles and examined under SEM (JSM5600, JEOL, Tokyo, Japan).

Results and Discussion

Several ultrastructural details of the external surface of the immature stages of *Lu. intermedia* and *Lu. whitmani* were revealed by SEM, allowing us to compare the microanatomy of these two important vectors.

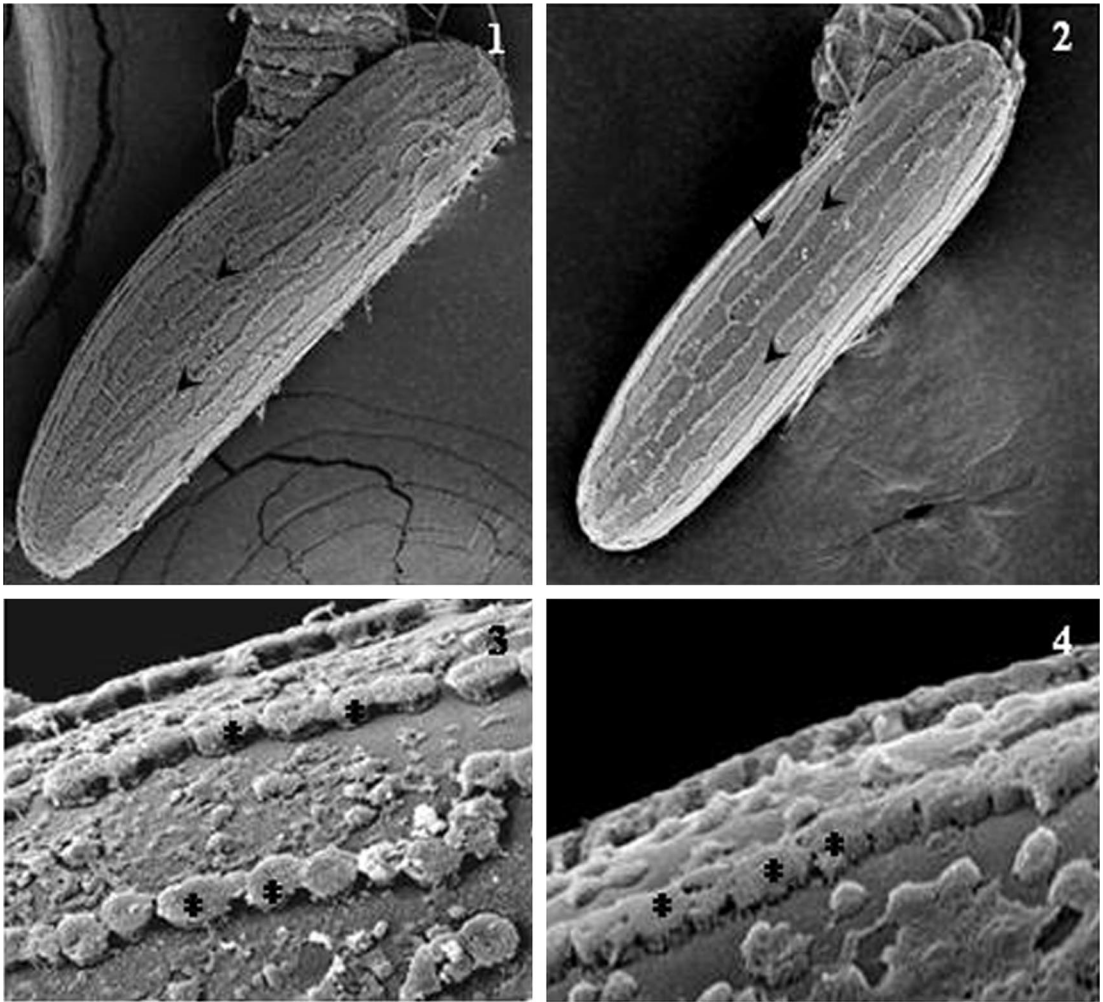
The eggs of *Lu. intermedia* and *Lu. whitmani* are about the same size (length 0.308 ± 0.016 mm and width 0.074 ± 0.003 mm for *Lu. intermedia*; length 0.307 ± 0.010 mm and width 0.077 ± 0.002 mm for *Lu. whitmani*), having a long, oval shape and ornamentations that consist of parallel ridges covering the entire exchorion (Figs. 1–4). Barreto (1941) described these ridges as “connected ridges” in these same species, by using optical microscopy. However, our SEM observations revealed that most of these ridges are unconnected (Figs. 1 and 2). Similar observations of unconnected ridges were reported by Almeida et al. (2004) on *Lu. intermedia* eggs. High SEM magnification revealed that these ridges are formed by single lines of

rounded palisade units (diameter 0.576 ± 0.074 μm for *Lu. intermedia* and 0.775 ± 0.095 μm for *Lu. whitmani*) that project from the surface of eggshell (high 0.454 ± 0.040 μm for *Lu. intermedia* and 0.597 ± 0.043 μm for *Lu. whitmani*) (Figs. 3 and 4). In contrast, *Lu. longipalpis* egg exchorions bear unconnected ridges formed by lines of double-rounded palisade units (Secundino and Pimenta 1999).

Some authors have speculated that exchorionic ornamentations are adaptive structures that facilitate oviposition in distinct environments (Ward and Ready 1975, Enrique Perez and Ogusuku 1997). In the present work, observations of the egg surfaces showed no differences in the exchorion ornamentations of the two sympatric sand flies. In another study, Rogo et al. (1992) showed intraspecific differences in the exchorions of *Phlebotomus aculeatus* (Lewis, Minter & Ashford 1974) and *Phlebotomus martini* (Parrot 1936). Yet another report showed that the egg exchorions of *Phlebotomus pedifer* (Lewis, Mutinga & Ashford), *Lu. longipalpis* and *Lutzomyia diabolica* (Hall 1936) have similar patterns of unconnected ridges (Endris et al. 1987, Rogo et al. 1992, Secundino and Pimenta 1999), even though these species occur in widely separated geographic areas. Others suggest, based on SEM examination of 23 sand fly species, that exchorionic ornamentations reflect phylogenetic relationships between the species (Endris et al. 1987, Feliciangeli et al. 1993, Enrique Perez and Ogusuku 1997, Fausto et al. 2001). Because the exchorionic ornamentation is not necessarily species specific, it is unlikely that it would be useful as a taxonomic tool.

Sand fly larvae escape from the egg by breaking the shell in a manner similar to other Diptera. The first sign of hatching is the appearance of a fracture toward the anterior end of the eggshell, which opens as a door-like flap through which the larva's head emerges (Figs. 5–7), revealing the egg burster (Fig. 8). Apparently, as the first instar moves its head inside the eggshell, this spine, located on the apex of the head capsule, scores the inside of the shell in an arc. Subsequent pressure applied by the larva causes the door-like fracture along the arc. As the head reaches outside the shell, the distal ends of the two caudal filaments also can be seen (Figs. 7–9). These sequential events allow the larva to escape (Figs. 9 and 10). A similar mechanism of egg breaking and larval escape was described for *Lu. longipalpis* (Leite and William 1997, Secundino and Pimenta 1999).

The breaking of the eggshell in several Diptera is due to the action of similar structures called egg bursters (synonyms hatching spine, egg-breaker, or bursting teeth). SEM showed the egg burster on the apex of the head of *Lu. intermedia* and *Lu. whitmani*. The egg burster exists only on the dorsal surface of the first instar head and has been used to differentiate the first instar from the second instar in Diptera, along with other taxonomic characters (Breland 1959, Alvan-Aguilar and Hamada 2003). Details of the microanatomy of the sand fly egg burster can be seen on the apex of head capsule when the larval head is completely out of the eggshell (Fig. 19). The egg bursters of these two

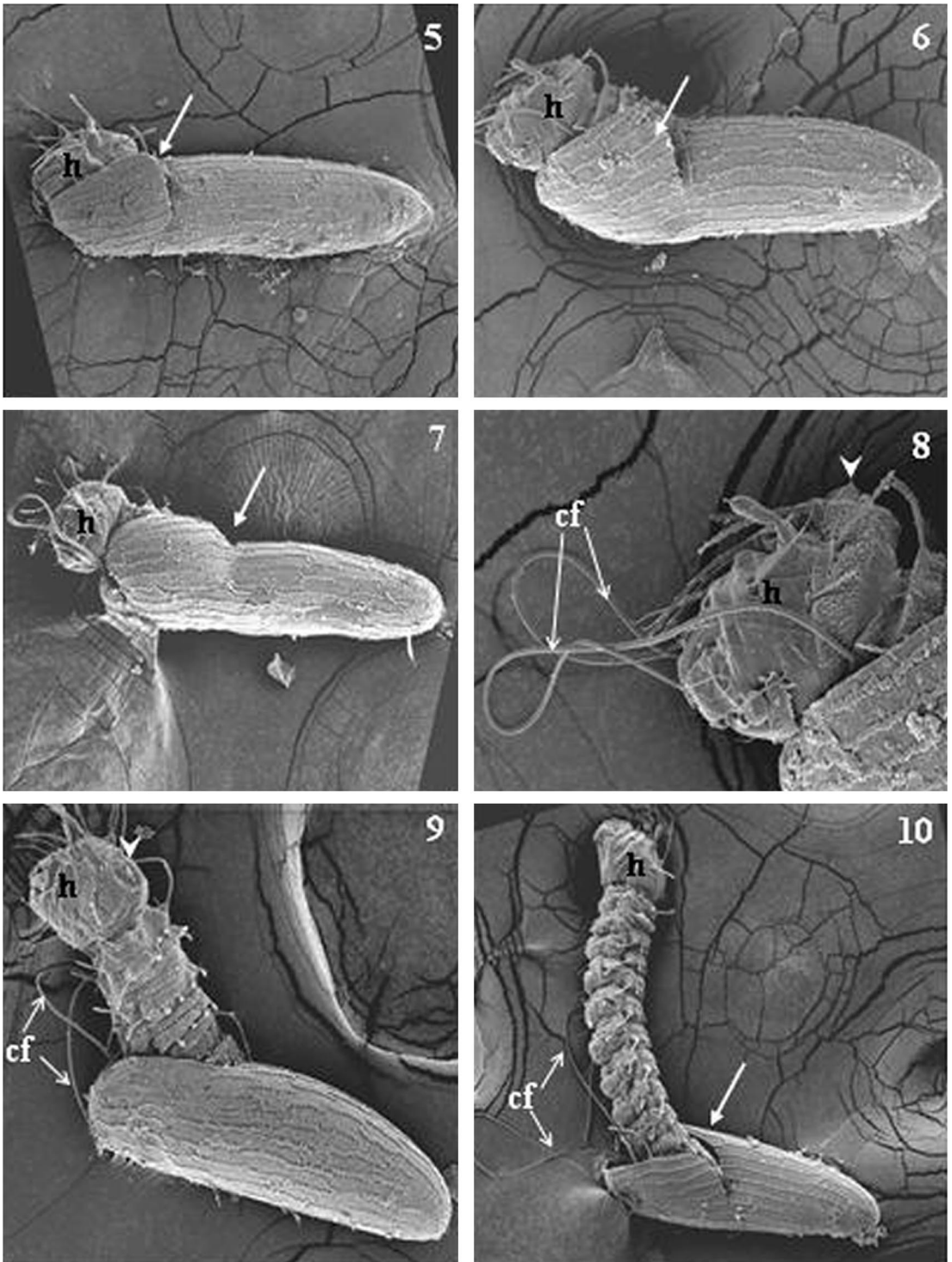


Figs. 1–4. Egg surfaces of *Lu. intermedia* (1 and 3) and *Lu. whitmani* (2 and 4) showing exchorionic ornamental unconnected parallel ridges (arrowheads). High magnification images (3 and 4) show that these ridges are formed by single lines of round palisade units (asterisks). Magnifications: 300 \times (1 and 2), 4,000 \times (3), and 3,000 \times (4).

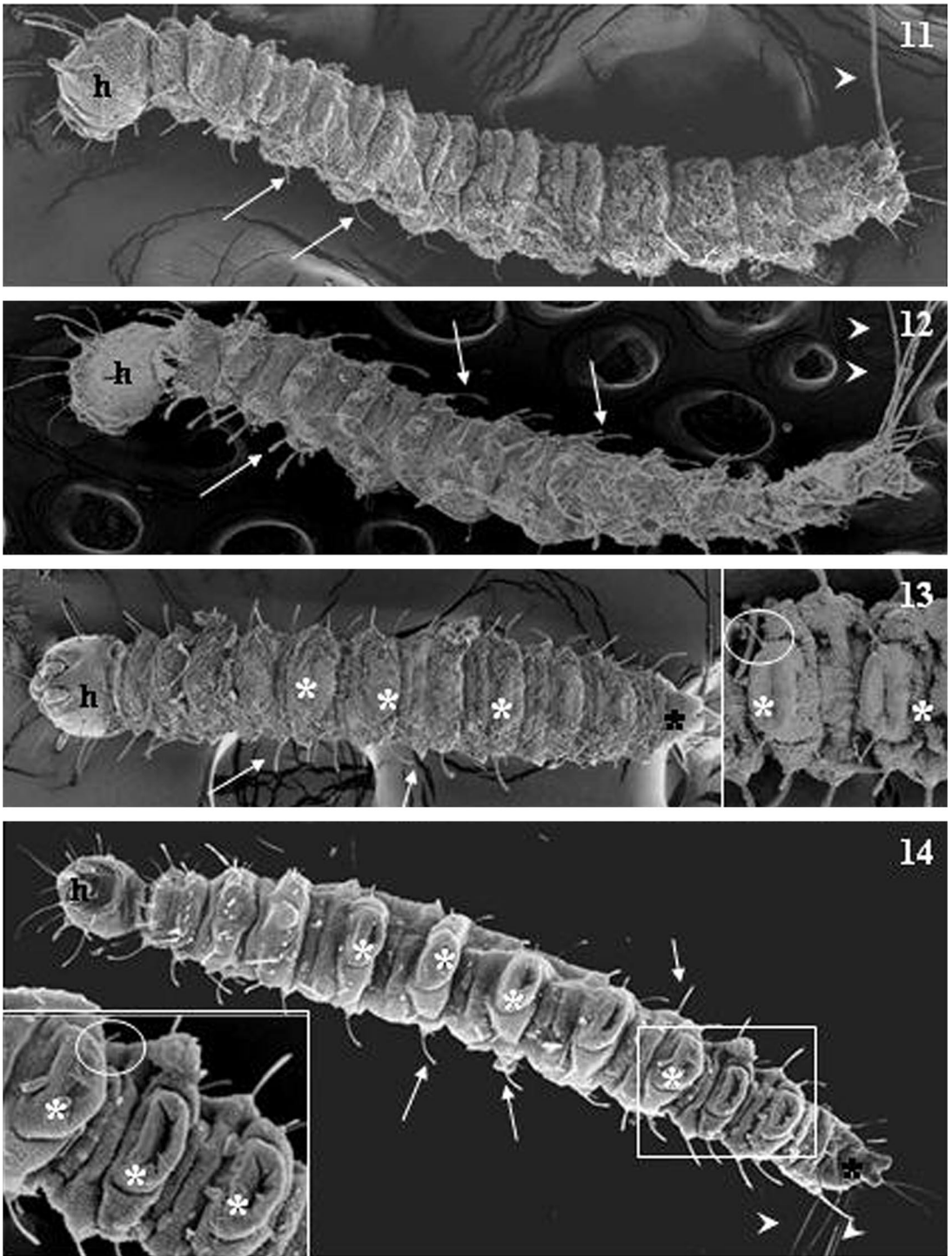
sand flies are similar structures with small differences in their dimensions (length 0.027 ± 0.003 mm and width 0.018 ± 0.001 mm for *Lu. intermedia* and length 0.023 ± 0.002 mm and width 0.019 ± 0.001 mm for *Lu. whitmani*). The egg burster is a cuticular formation that rises from the top of the larva's head like a miniature volcanic cone (Fig. 19). Leite and William (1997) and Secundino and Pimenta (1999) observed a similar egg burster in *Lu. longipalpis*.

The general microanatomy revealed by SEM of the larvae of both sand fly species is similar. The bodies of both larvae are long and divided into 12 segments: three thoracic and nine abdominal segments (Figs. 11–14). In both species, the larval integument is adorned dorsally and laterally with numerous setae (Figs. 11–14), except on the anal lobe (Figs. 15 and 16), on the head base and on the mouthparts (Figs. 17–19). The abdominal segments have prolegs (protruding elliptical structures) ventrally that are used for

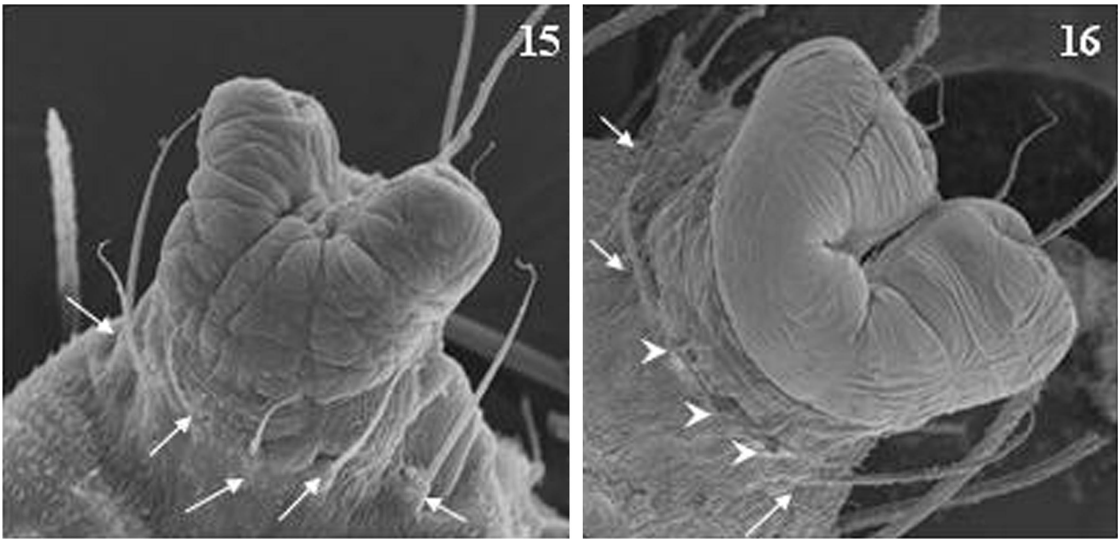
locomotion and are not present on the first three segments nor on the anal lobe (Figs. 13 and 14, insets). These anatomical aspects are similar in *Lu. longipalpis* and *Lutzomyia bahiensis* (Mangabeira & Sherlock 1964) larvae (Sherlock and Carneiro 1963; Leite and William 1996, 1997; Secundino and Pimenta 1999). *Lu. intermedia* and *Lu. whitmani* prolegs have small differences in size (length 0.068 ± 0.001 mm and width 0.030 ± 0.004 mm for *Lu. intermedia*; length 0.108 ± 0.005 mm and width 0.057 ± 0.006 mm for *Lu. whitmani*). In addition, the morphology of the anal lobe is different between the species (Figs. 15 and 16). The anal lobe of *Lu. whitmani* is more wrinkled and shorter than that of *Lu. intermedia* (length 0.114 ± 0.002 mm and width = 0.123 ± 0.003 mm for *Lu. intermedia*; length 0.067 ± 0.006 mm and width 0.076 ± 0.006 mm for *Lu. whitmani*). These characteristics may comprise distinguishing features that might be useful to confirm the identification of these



Figs. 5-10. Sequential events of egg hatching of *Lu. intermedia* and *Lu. whitmani*. Figures 5 and 6 show a door-like flap at the anterior end of the eggshell (arrow), where the larval head first emerges. The entire head (h) is exposed outside the eggshell in 7 and 8. In 8 it is possible to see the egg burster (arrowhead) on the larva's head and the distal ends of the caudal filaments (cf). 9 and 10 show, respectively, two larvae, one larva halfway and larva almost completely emerged from the eggshell. Note the egg burster (arrowhead) on the larva's head. Magnifications: 270 \times (5), 330 \times (6), 230 \times (7 and 9), 550 \times (8), and 190 \times (10).



Figs. 11–14. External morphology of *Lu. intermedia* (11 and 13) and *Lu. whitmani* larvae (12 and 14). Lateral views of the sand fly larvae (11 and 12) show the heads (h) and the caudal filaments (arrowheads). Ventral views (13 and 14) show the larval bodies divided into 12 segments, three thoracic and nine abdominal segments (white asterisks on second, third and fifth abdominal segments) between the head and the anal lobule (black asterisks). Note the prolegs (white asterisks in 13 and 14) are only present on the abdominal segments. Details of the prolegs and curved long trichoid sensillae (circle) are seen in the two insets. The brush-like trichoid sensillae (arrows) are located in dorsal and lateral parts of larval segments. Magnification: 220× (11), 60× (12), 160× (13, and inset, 500×), and 35× and inset, 250×).



Figs. 15–16. Details of the larvae's anal lobe. In *Lu. whitmani* (15) this structure is more wrinkled and shorter than in *Lu. intermedia* (16). Note the crowns of short and long trichoid sensillae (arrows) surrounding the base of the anal lobes. Some sensillae were accidentally removed during sampling process and it is possible to see their insertions sites (arrowheads). Magnifications: 500 \times (15) and 400 \times (16).

species under SEM, further supporting ordinary adult taxonomy.

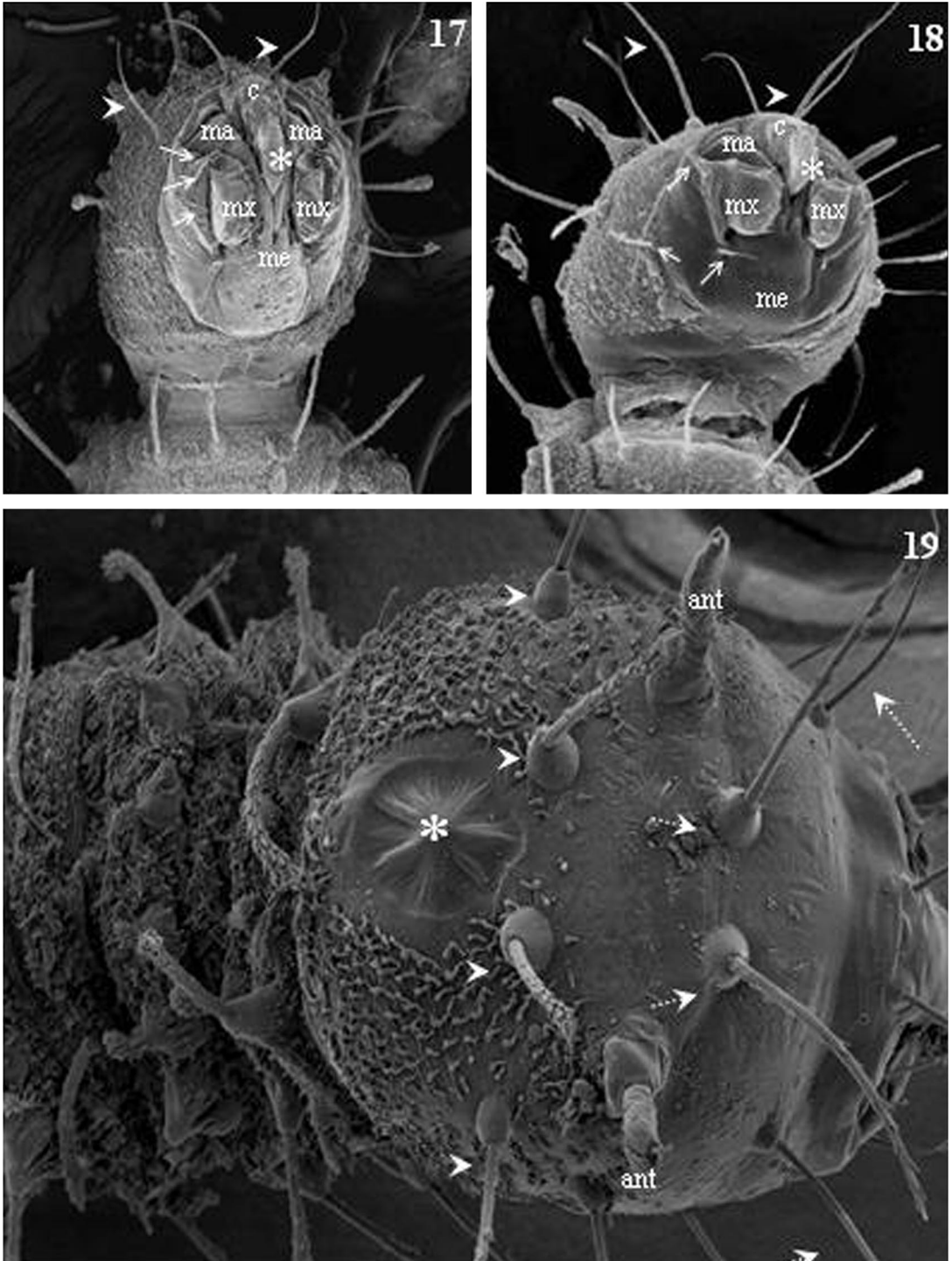
The larvae of both species have well-defined, round, and sclerotized head capsules (Figs. 11–14 and 17–19). The mouth apparatus, consisting of clypeus, labrum, mandibles, maxillae, and mentum, located ventrally on the head, measures 0.147 ± 0.025 mm in length and 0.135 ± 0.025 mm in width in *Lu. intermedia* and 0.092 ± 0.032 mm and 0.091 ± 0.027 mm in *Lu. whitmani* (Figs. 17–18). This “masticator”-type mouthpart apparatus was described by Leite and William (1996, 1997) and Mukhopadhyay and Ghosh (2000).

Antennal morphology is similar in *Lu. intermedia* and *Lu. whitmani* and changes between the L1 and subsequent instars (L2, L3, and L4). The L1 antennae are two-segmented with a basal conical segment fused with a short ovoid distal segment that is almost as long as the basal segment (Figs. 19–21). In the L2 through L4, the antennae are also two-segmented, but the distal segment is greatly elongated (digitiform) to ≈ 3 times the length of the conical basal segment (Figs. 22–23). This observation differs from the initial findings of Barreto (1941), who observed digitiform antennae on all larval instars of both species using optical microscopy. Recently, Pessoa et al. (2001) described similar digitiform antennae on the L4s of several sand fly species.

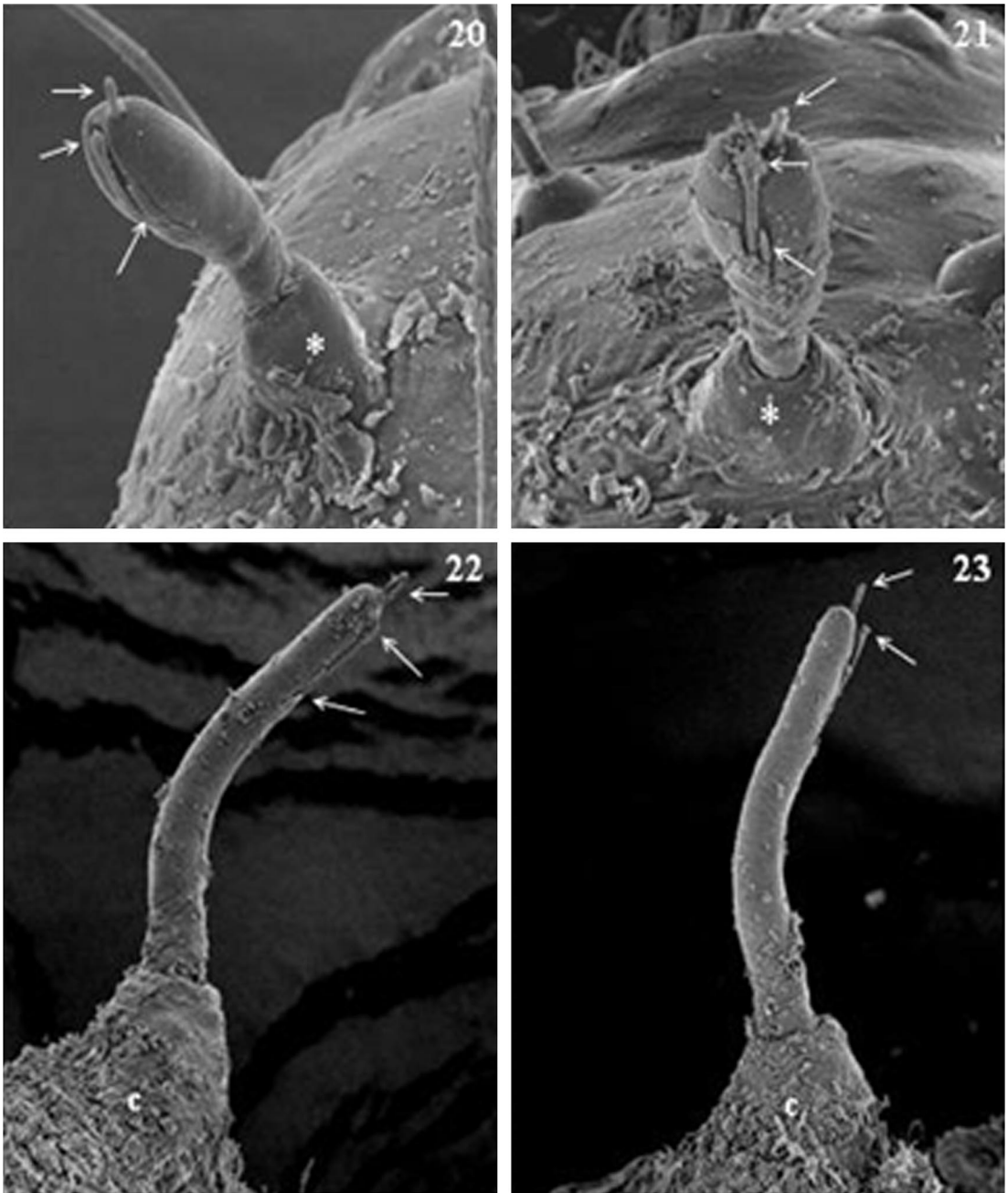
We analyzed in detail all the sensilla types present on the immature stages of *Lu. intermedia* and *Lu. whitmani*, categorizing them according to the classification scheme of Hallberg and Hansson (1999) and Mitchell et al. (1999). There are five subtypes of trichoid sensillae: 1) long, 2) short, 3) curved long, 4) brush-like, and 5) weakly brush-like. Long trichoid sensillae (0.066 ± 0.011 mm in length for *Lu. intermedia* and 0.068 ± 0.022 mm in length for *Lu. whitmani*)

are located on the forehead in a horizontal row in front of the weakly brush-like trichoid sensillae (Figs. 17–19, 28, and 29). Short trichoid sensillae (0.021 ± 0.010 mm for *Lu. intermedia* and 0.028 ± 0.012 mm for *Lu. whitmani*) were observed on the mouthparts (Figs. 17 and 18 and 28). Long and short trichoid sensillae ring the anal lobes in both species (Figs. 15 and 16). This type of sensillum was not found in *Lu. longipalpis* and *Lu. bahiensis* (Sherlock and Carneiro 1963; Leite and William 1996, 1997; Secundino and Pimenta 1999). The curved long trichoid sensillae, which exhibit varying degrees of curvature, are located on the lateral sides of the prolegs in both species (Figs. 13 and 14, insets; 27 and 28). These were not observed by Sherlock and Carneiro (1963) in *Lu. bahiensis* or by Leite and William (1996, 1997) and Secundino and Pimenta (1999) in *Lu. longipalpis*. Straight and curved forms of long trichoid sensillae and short trichoid sensillae were observed previously by Fernandes and Linardi (2002) in segment II of the mouthparts of *Dermatobia hominis* (L. Jr. 1781) (Diptera: Oestridae). Mechano-sensory sensillae with similar morphology have been found in the maxillary palps of *Drosophila melanogaster* Meigen by Riesgo-Escovar et al. (1997). Brush-like trichoid sensillae are localized along the dorsal and lateral surfaces of the larval body, but they are absent on the ventral surfaces, and behind the antennae on the top of the head (Figs. 11–14, 19, 24–25). Weakly brush-like trichoid sensillae are found in the middle of the head in front of the antennae (Figs. 19 and 26).

Detailed examination of the larval antennae revealed three types of sensillae on the ventral and apical surfaces of the terminal segment, present in all instars regardless of the changes in antennal morphology from L1 to L2–L4 (Fig. 30). These sensillae are



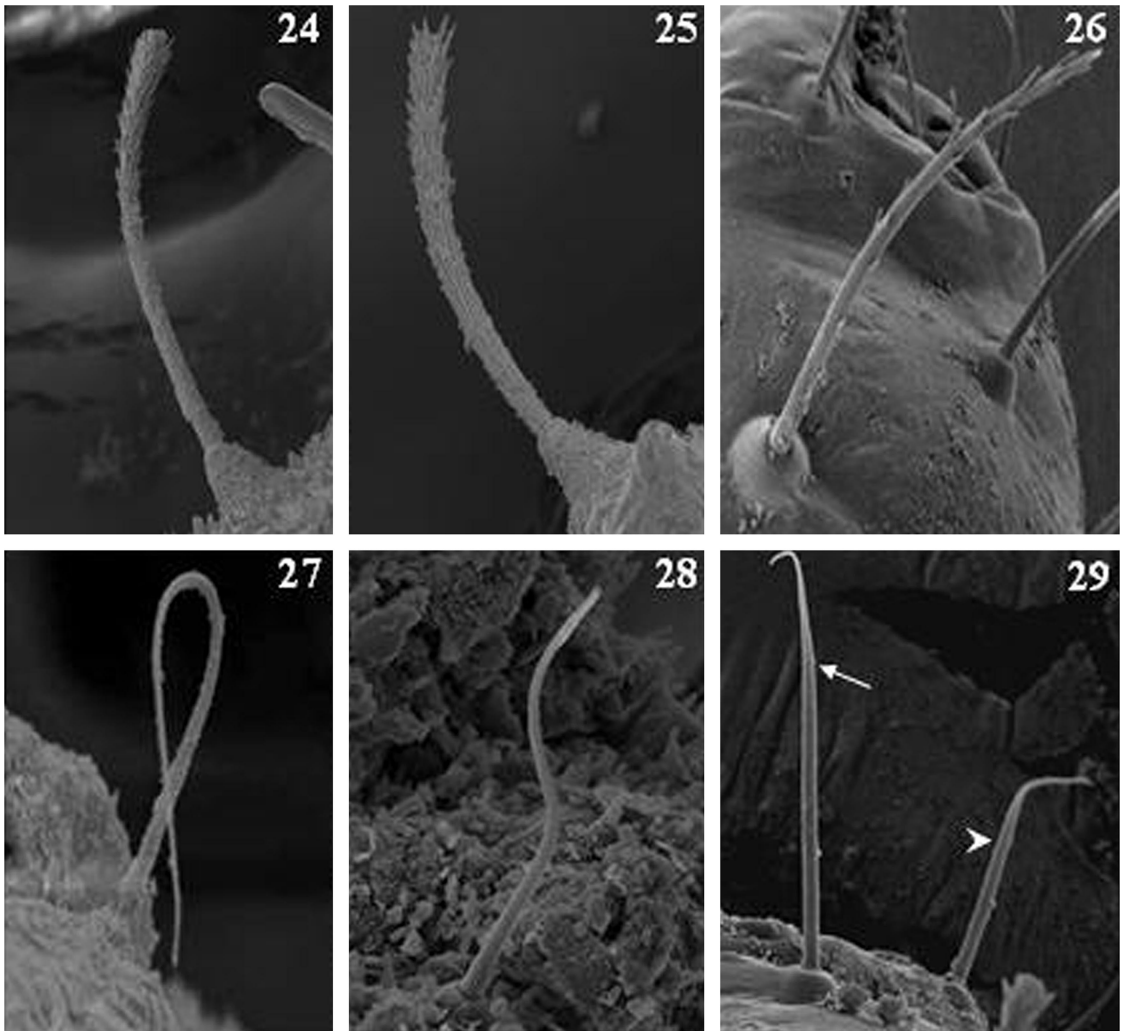
Figs. 17–19. External morphology of the larval heads of *Lu. intermedia* (17 and 19) and *Lu. whitmani* (18). Ventral views of the heads show the mouth apparatus consisting of clypeus (c), labrum (asterisks), mandibles (ma), maxillas (mx), and mentum (me). Note trichoid sensillae on the apex of the head (arrowheads) and the short trichoid sensillae on the mouth apparatus (arrows). Dorsal view of the head (19) shows the egg burster (asterisk) as a “volcanic cone” structure. A row of brush-like trichoid sensillae (arrowheads) is located in front of the egg burster (asterisk). On the forehead are two weakly brush-like trichoid sensillae (small arrows) inserted slightly forward and between the antennae (ant) and long trichoid sensillae (large arrows) are inserted further down toward the mouth. Magnifications: 350× (17), 300× (18), and 1,000× (19).



Figs. 20–23. Antennal morphology in larvae of *Lu. intermedia* (20 and 22) and *Lu. whitmani* (21 and 23). The antennae of L1 are two-segmented with a basal conical segment fused with a short ovoid distal segment (asterisks) (20 and 21). The antennae in L2s through L4s are also two-segmented, but the distal segment is greatly elongated (digitiform) (c) (22 and 23). Note the sensillae inserted on the superior portion of the antennae (arrows). Magnifications: 2,000 \times (20 and 21), 1,000 \times (22 and 23).

important sensory organs that function as hygroreceptors, contact chemoreceptors, tactile receptors, and olfactory receptors and temperature receptors, enabling the insects to detect changes in the surrounding environment. Other sensillae are found on the antennae of *Lu. whitmani* and *Lu. intermedia* larvae.

The first of these is a clavate-basiconic sensillum located at the apex of the segment (Fig. 30). At the proximal end of this antennal segment are three short, blunt, coeloconic sensillae inserted at the base of a single large, multiporous, clavate-coeloconic sensillum (Figs. 30 and 31). Olfactory sensillae with bul-

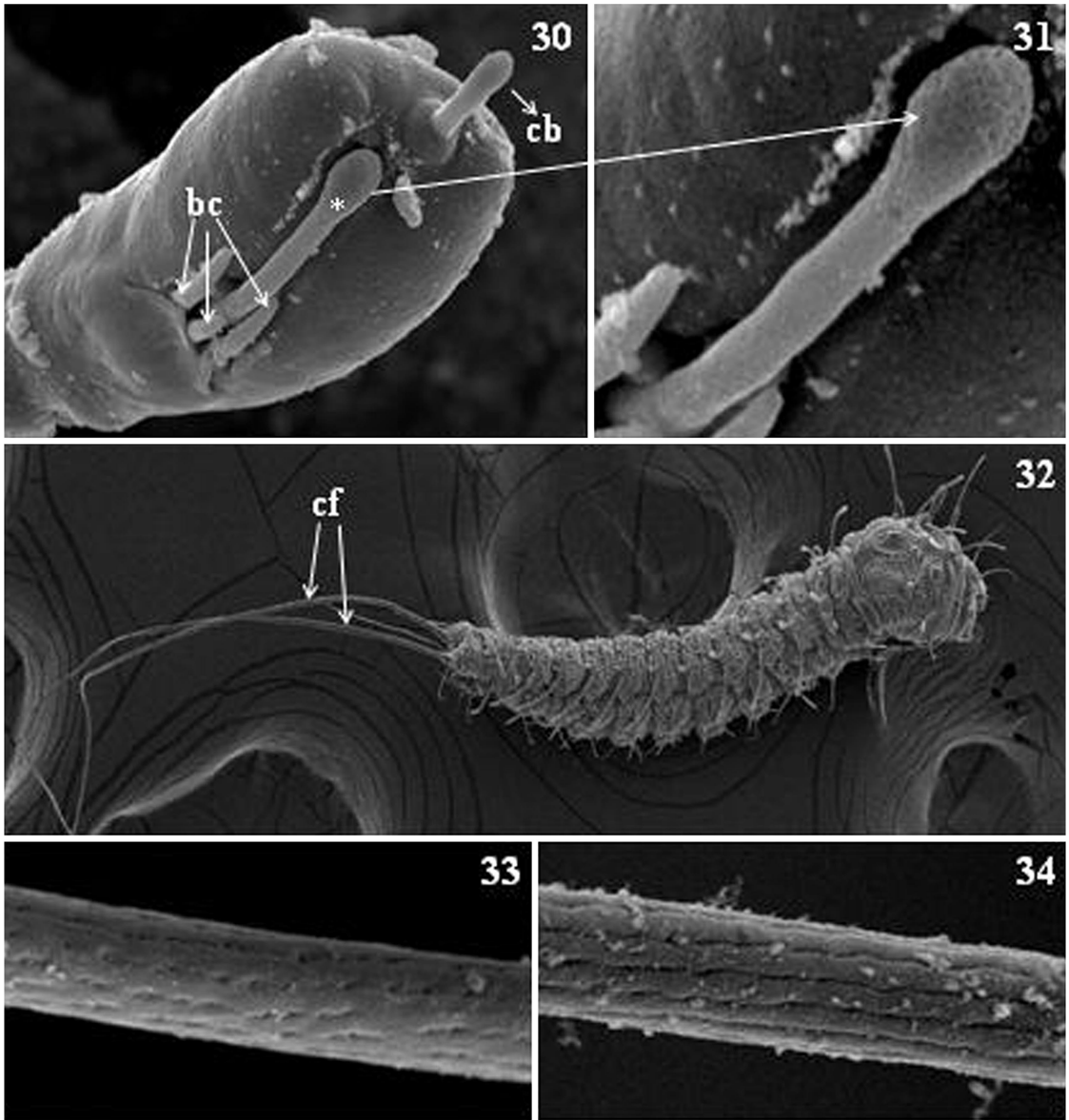


Figs. 24–29. Microanatomy of the sensillae of *Lu. intermedia* and *Lu. whitmani* larvae showing brush-like trichoid sensilla present on the head (24) and on the lateral and dorsal aspects of the body segments (25), weakly brush-like trichoid sensilla on the middle of the head (26); curved long trichoid sensillae with different degrees of bending on the lateral sites of the prolegs (27 and 28); and short trichoid sensillae (arrowhead) and long trichoid sensillae (arrow) on the top of the larva's head (29). Magnifications: 1,000 \times (24 and 25), 2,000 \times (26, 27, 29), and 3,000 \times (28).

bous, porous apical dilations (clavate sensillae), similar to the multiporous clavate-coeloconic sensilla described here, were observed on the antennal clavae of *Trichogramma galloi* (Zucchi 1988) and *Trichogramma pretiosum* (Riley 1879) (Hymenoptera: Trichogrammatidae), on the maxillary palps of *Culicoides hollensis* (Melander & Brues 1903) and *Culicoides melleus* (Coquillett 1901) (Diptera: Ceratopogonidae), and on the antennal funiculus of *Cochliomyia hominivorax* (Coquerel 1858) (Diptera: Calliphoridae) (Consoli et al. 1999, Kline and Axtell 1999, Fernandes et al. 2004).

Another long type of trichoid sensillum with wall pores is located on the last larval segment (Fig. 32). In sand flies, sensillae of this type are called caudal filaments. There are two caudal filaments on the L1 and four on the L2 through L4. Different patterns of pores

exist on the caudal filaments of the two species studied. *Lu. whitmani* has pores situated inside the wall grooves (Fig. 34). In *Lu. intermedia*, the grooves are not as deep as in *Lu. whitmani*, and the pores occur to be on the surface (Fig. 33). Pessoa et al. (2001) found pores on caudal filaments of larvae of *Lu. intermedia*, *Lu. whitmani*, *Lutzomyia lenti* (Mangabeira 1938), *Lu. longipalpis*, and *Lutzomyia evandroi* (Costa Lima & Antunes 1936), but they did not find any differences in the pattern of pores between the first two species. Zacharuk (1985) suggests that the absence of pores on the sensorial cuticle does not allow chemical sensitivity and that the presence of a single pore limits the sensitivity to taste, whereas several pores allow the sense of olfaction. According to Zacharuk, the caudal filaments and the multiporous clavate coeloconic sensillae of the first instar of *Lu. intermedia* and *Lu. whit-*



Figs. 30–34. Microanatomy of the antennae and caudal filaments of *Lu. intermedia* (30 and 31, 32 and 33) and *Lu. whitmani* (30 and 31 and 34) larvae. Image 30 shows a general view of the antenna of *Lu. intermedia* L1 larva (similar to *Lu. whitmani*). Note five sensillae: one clavate basiconic sensilla (cb) on the apex, one multiporous clavate coeloconic sensilla (asterisk) on the base, and three short blunt coeloconic sensillae (bc) on the proximal region. Observe the pores on the clavate coeloconic sensillum at a higher magnification (31). Image 32 shows the general view of the caudal filaments (cf) (i.e., long type of trichoid sensillae) of *Lu. intermedia* L1, which are similar in *Lu. whitmani* (not shown) at low magnification. Pores in the caudal filaments are only seen at high magnifications. In *Lu. whitmani*, the pores are inside wall grooves (34), whereas in *Lu. intermedia* (33) the pores are revealed in the surface because the grooves are not so deep. Magnifications: 9,000 \times (30), 21,600 \times (31), 150 \times (32), 6,000 \times (33), and 8,000 \times (34).

mani respond to olfactory molecules; thus, they are considered chemoreceptors.

In this study, we found mainly trichoid sensillae on the larval segments of both sand fly species studied. Green and Hartenstein (1997) analyzed the sensillar patterns on the thoracic and abdominal segments of several insect orders and described hairs and bristles

(trichoid and chaetic sensillae) of varied sizes and number in primitive insects and peg-like sensillae (basiconic, styloconic, and coeloconic) and papilla sensillae in Diptera. These authors suggested that the trichoid sensillae represent mechanoreceptors that are stimulated by hair deflection. However, Zacharuk (1985) claims that trichoid sensillae can have various

functions, including mechanical, chemical, and thermal reception that enable the larvae to discern changes in their environment. Future ultrastructural and electrophysiological studies will be necessary to determine the exact function of these sensillae in sand flies. Added to the results of the present work, these studies may supply information about the behavior and physiology of these important vectors that will be useful in the development of vector control strategies.

In summary, our comparative study showed that the external morphology of the eggs and larvae of *Lu. intermedia* and *Lu. whitmani* have similar features but are distinguishable by details in their microanatomy. The similarities found between these two species most likely reflect their close phylogenetic relationship.

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