Scanning Electron Microscopic Study of the Egg and Immature Stages of the Sandfly Lutzomyia longipalpis.

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Abstract

The morphology of the egg, larva and pupa of the sandfly *Lutzomyia longipalpis*, vector of *Leishmania chagasi*, was studied by means of scanning electron microscopy. The eggs had patterned exochorion, while the segmented bodies of the larva and pupa showed their surfaces covered by minute spines, hairs or pseudo-legs according to the observed region. Details of the head, mouth parts, and the caudal segment are also described. This study provides basic information for further comparative studies with other *Lutzomyia* species.

Keywords: SEM - eggs - immature stages - *Lutzomyia longipalpis*

Running title: SEM of *Lu. longipalpis* Immature Stages.

Introduction

Lutzomyia longipalpis is a New World phlebotomine sand fly responsible for transmission of *Leishmania chagasi* causative agent of visceral leishmaniasis in Brazil and other South American countries. The life cycle of *Lutzomyia* spp. sandflies in the wild is still unknown but the colonization of some species has allowed the immature stages to be studied in the laboratory. The insect has four developmental stages: egg, larva, pupa and adult. The first description of egg, fourth instar larva and pupa of *Lu. longipalpis* by optical microscopy was done by Guitton and Sherlock (6). Following this study, scanning electron microscopy was used to reveal details of the immature stages of distinct species of *Lutzomyia* with descriptions of the eggs (17), pupae (11) and first and fourth stages larvae (12, 13).

Recent biochemical and molecular studies indicate that *Lu. longipalpis* is a complex of species distributed in different regions of America with at least three sub-species (8) presenting distinct chromosomal patterns (16), but there is no morphological evidence until now. The objective of this SEM study was to describe and illustrate morphological details of all immature stages (egg to pupa) during the development of *Lu. longipalpis* in a colony recently established from Minas Gerais state, Brazil. The results provide the basis for future comparative morphological studies with insects collected in different geographic regions in order to provide further evidence that *Lu. longipalpis* is a complex of species.

Material and Methods

Sandfly - Eggs, larvae and pupae of *Lu. longipalpis* were obtained from a closed laboratory colony established from flies collected in Lapinha Cave, Minas Gerais State, Brazil, and reared at the Laboratory of Medical Entomology of the Centro de Pesquisas René Rachou - Fiocruz, following the modified method described by Modi (9).

S E M - Eggs and larvae were rapidly washed in PBS (Phosphate Buffered Saline) treated with 0.25% trypsin/ 0.02% Versene solution for 5 min and fixed overnight in Karnovsky's fixative - 4% glutaraldehde and 5% formaldehyde in 0.1 M cacodylate buffer at pH 7.2 (7). The fixed material was rinsed in buffer, dehydrated in a graded ethanol series, dried by the critical point method using liquid $C0_2$, coated with gold and observed by scanning electron microscopy (SEM).

Results and Discussion

The insect eggs are long, oval and curved structures of in length 1.7 mm and 0.67 mm wide (Figs. 1 and 2). The SEM showed morphological details of the eggshell, the chorion, which is constituted by the exochorion, the outer surface in

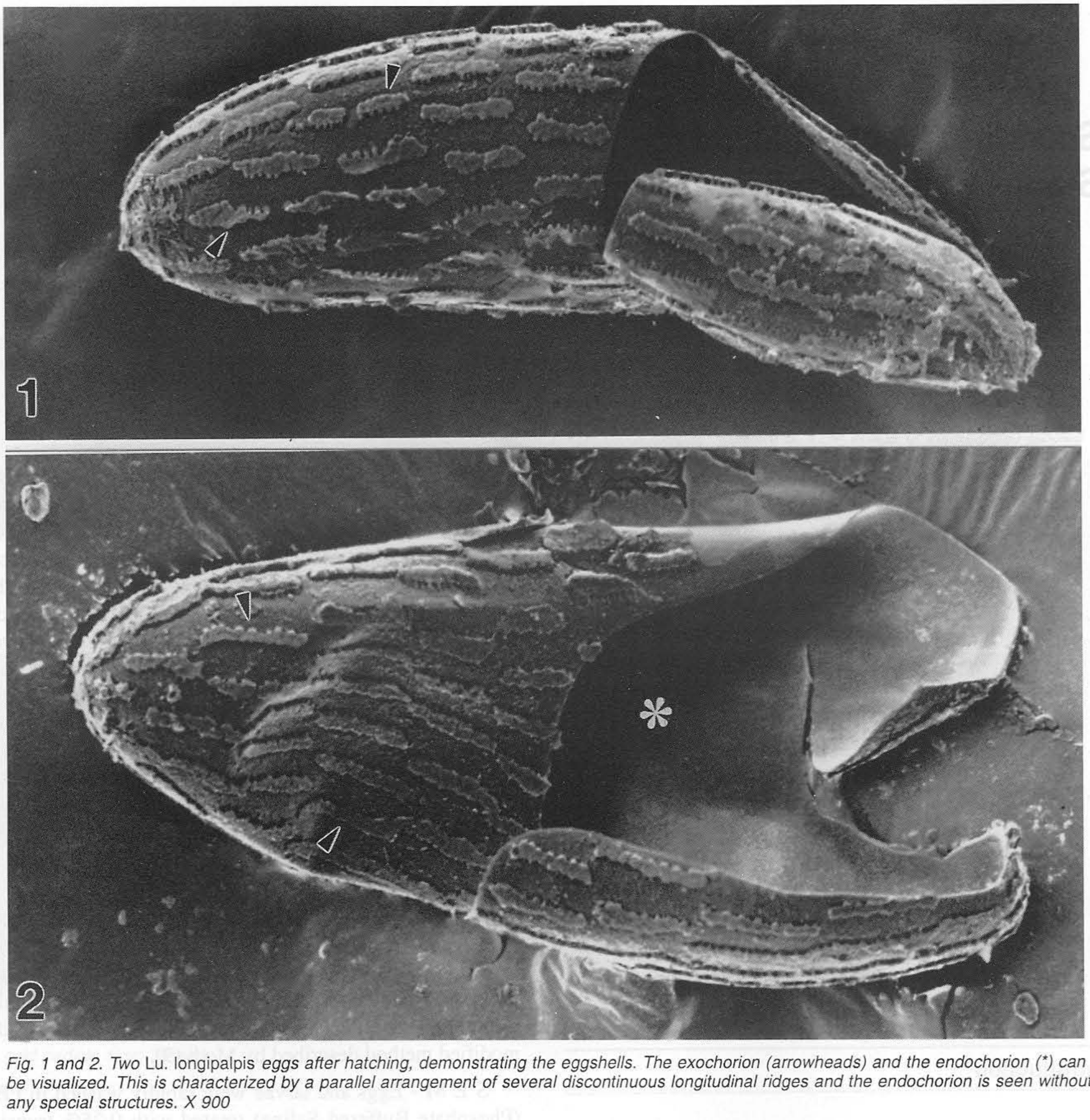

Fig. 1 and 2. Two Lu. longipalpis *eggs after hatching, demonstrating the eggshells. The exochorion (arrowheads) and the endochorion (*) can be visualized. This is characterized by a parallel arrangement of several discontinuous longitudinal ridges and the endochorion is seen without any special structures. X 900*

contact with the environment; and the endochorion, the inner surface that envelopes the embryo (Figs. 1-3). The exochorion is characterized by a parallel arrangement of several discontinuous longitudinal ridges at a distance of 4.62 mm from each other in the middle of the egg. There are no lateral connections between the ridges but all converge at the ends. A high magnification of the egg surface in the SEM (Fig. 3) revealed that the ridges are formed by palisade structures of 0.8 mm width, projecting from the eggshell. The base of these structures are inserted in the chorion. The exochorion surface between the palisades had numerous small rounded protuberances. There was no morphological differences between the sculptures in the dorsal and the ventral surfaces. The observation of cracked shells of broken and hatched eggs made possible to visualize the endochorion throughout the openings of the eggshell (Fig. 2). The endochorion is characterized by a smooth surface with no visible structures.

The eggshell of insects including sand flies has the function of enclosing and protecting the embryo. The pattern of the of *Lutzomyia* egg exochorion, usually showing an elaborated sculpted surface has been described in several species. The first observations were made using optical microscopy (2, 4, 14, 15). Later, use of SEM allowed the visualization of sculptural arrangements of the exochorion and their characterization in 40 *Lutzomyia* species (3, 4, 5, 10, 17, 18). These studies showed projections out of the surface forming distinct arrays in the exochorion of different species of

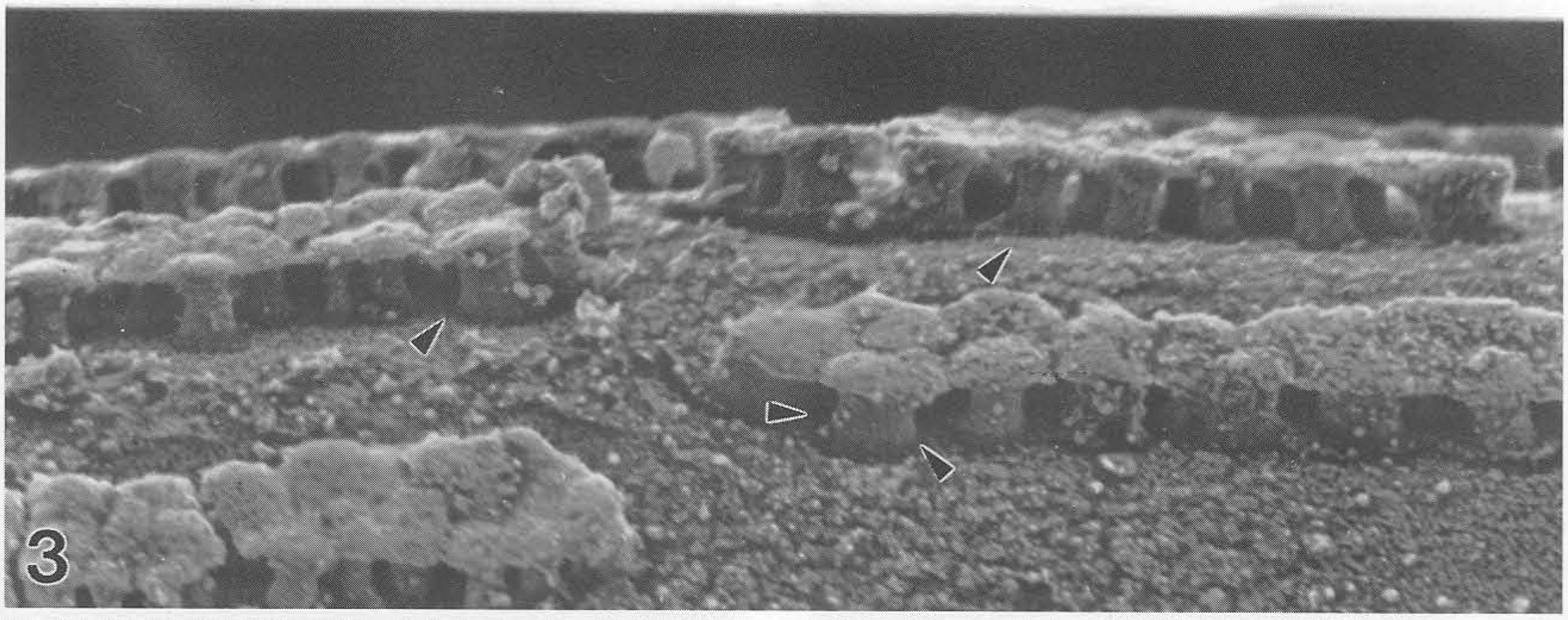

Fig. 3. Large magnification of the eggshell, showing details of the parallel ridges of the exochorion. The ridges are constituted by arrangements of palisade structures (arrowheads). X4,500

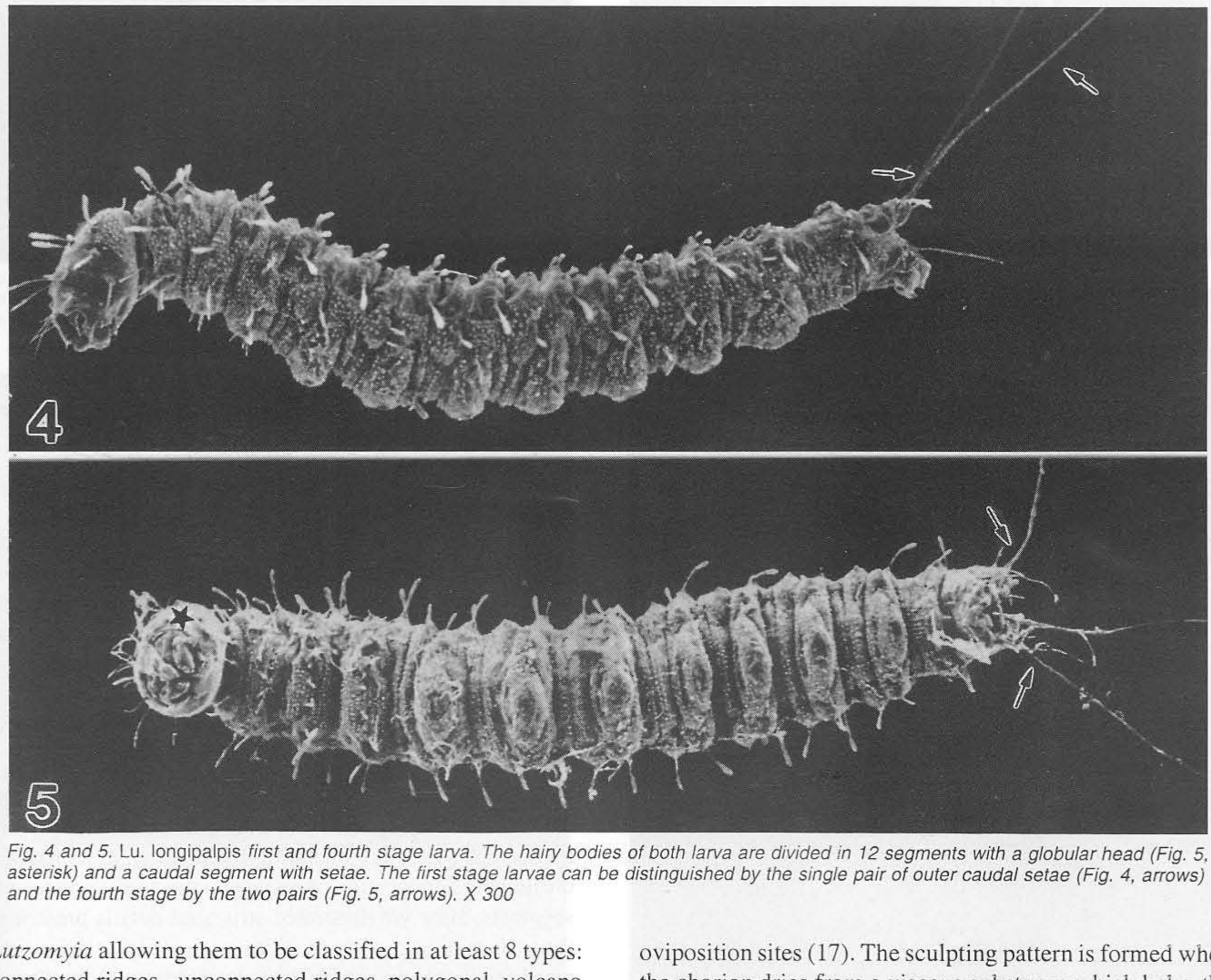

Fig. 4 and 5. Lu. longipalpis *first and fourth stage larva. The hairy bodies of both larva are divided in 12 segments with a globular head (Fig. 5, asterisk) and a caudal segment with setae. The first stage larvae can be distinguished by the single pair of outer caudal setae (Fig. 4, arrows) and the fourth stage by the two pairs (Fig. 5, arrows). X 300*

Lutzomyia allowing them to be classified in at least 8 types: connected ridges, unconnected ridges, polygonal, volcano or mountain like, elliptical, reticular , verrucose and disperse. All these studies suggested that the topographic patterns of the egg exochorion reflect phylogenetic relationships. There is evidence supporting the idea that they may represent specific adaptations to the environmental conditions in oviposition sites (17). The sculpting pattern is formed when the chorion dries from a viscous substance which helps the eggs stick to substrates. According to our observations, *Lu. longipalpis* is included in the group that presents unconnected parallel ridges. The same pattern was also seen in *Lu. diabolica*, *Lu. renei*, *Lu. whitmani* and *Lu. pestani* (1, 3,15,17),demonstrating that distinct sandfly species can have

the same sculpting pattern. However, diverse exochorion patterns have been observed in eggs from different sandfly populations of *Lu. verrucarum* in Andean valleys (10), suggesting intraspecific variation.

The *Lu. longipalpis* first instar larva is 0.47 mm long and 0.06 mm wide (Fig. 4). It is the smallest larva and is distinguished from latter instars by the presence of a single pair of outer caudal setae, the other stages having two pairs. The fourth stage larva, the last step before pupation, is 3.1 mm long and 0.42 mm wide (Fig. 5). With the exception of differences in the number of caudal setae pairs, the general aspect of all larvae in different stages is very similar. The body is divided into twelve segments, three thoracic and nine abdominal, with a globular head (Fig. 8) and a caudal segment with two pairs of long outer caudal setae (Fig. 9). SEM showed the integument of the dorsal and lateral regions of larva body to be covered by arrow-shaped hairs of uniform length (Figs. 5, 8 and 11). These hairs are 7 mm long and have branches that increase in size from the base upward giving them the differentiated arrow-like shape (Fig. 11). There are no hairs in the larva ventral surface which is characterized by presence of bulbous structures projecting from the integument in each segments, the pseudo-legs, which are used by larva locomotion (Fig. 10). Large magnifications showed the larval body surface to be completely covered by numerous minute spines of uniform size, having

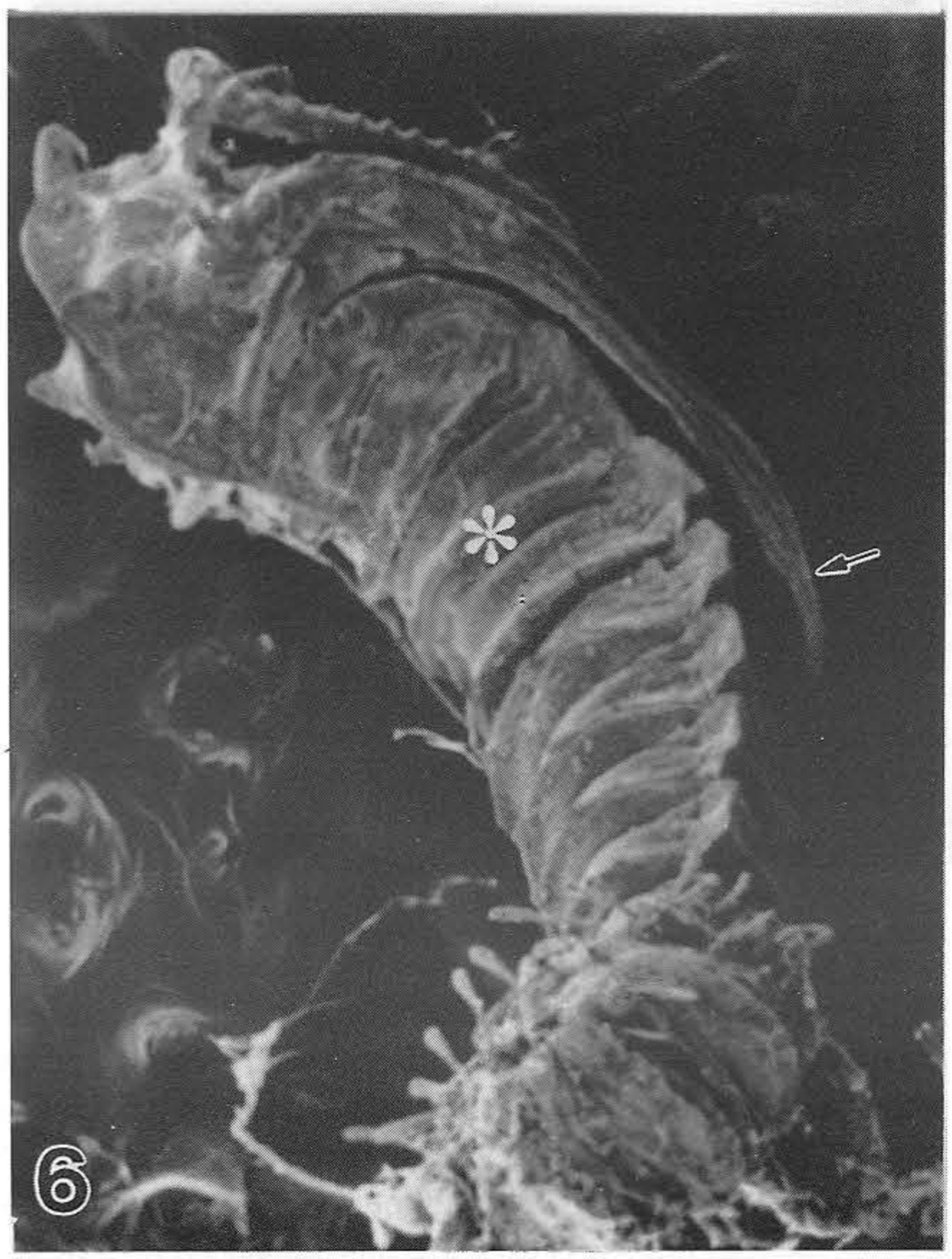

Fig. 6. Lu. longipalpis *pupa. The pupa has elliptical shape with a segmented body (*). It is possible to observe the adult's future wings (arrow).X 180*

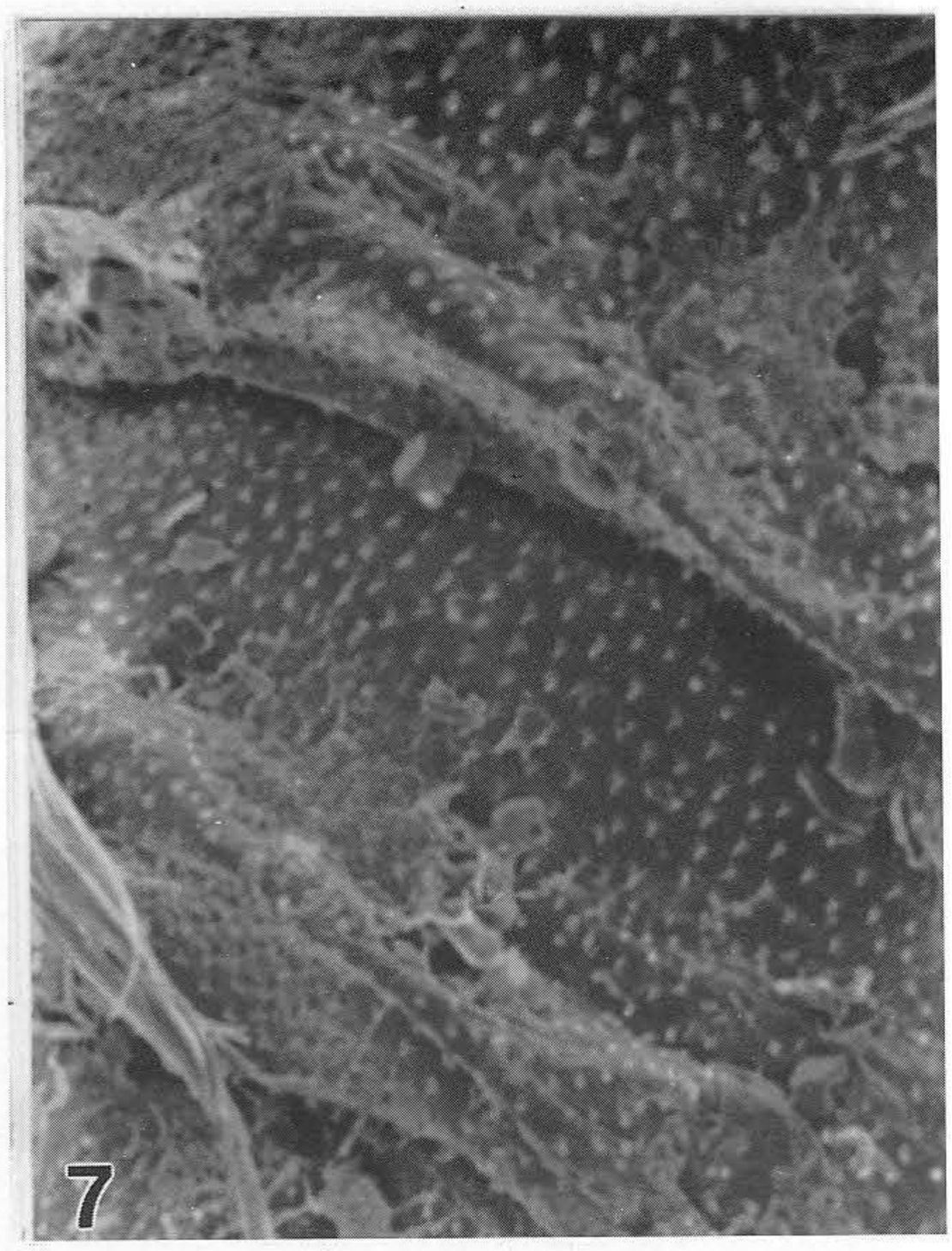

Fig. 7. Large magrification of the pupa body. The body surface is completely covered by minute spines. X 1,300

regular transversal alignments over the integument, except in the regions at the base of the head (Fig 8) and in the pseudo-legs (Fig. 10).

The *Lu. longipalpis* pupa is elliptical shape with a length of 2.45 mm and the greatest width of 0.7 mm decreasing to 0.3 mm at the posterior end, which lies within the exuvium of the last larval instar that maintains the insect attached to a substrate (Fig. 6). Throughout the pupa integument it is possible to visualize the adult segmented body indicating head, eyes and wings. The pupa integument is completely covered by minute spines similar to these of the larva (Fig 7) except in the head where a projecting pair of antenna can be seen (Fig 6).

SEM studies have revealed aspects of the immature stages of some sand fly species (12, 13). These studies deal with number of structures including mouthparts, antennae and the number and location of hairs over the body surfaces and segments. Here we illustrated structural details present on the surface of eggs and immature stages of laboratory-colonized *Lu. longipalpis* sand flies collected from Minas Gerais, Brazil. This data can be used as a basis for distinguishing the insect from different species comparing populations collected in different geographic regions. It may be possible to find morphological markers to support the idea of the existence of complex ratter than a single species classified as

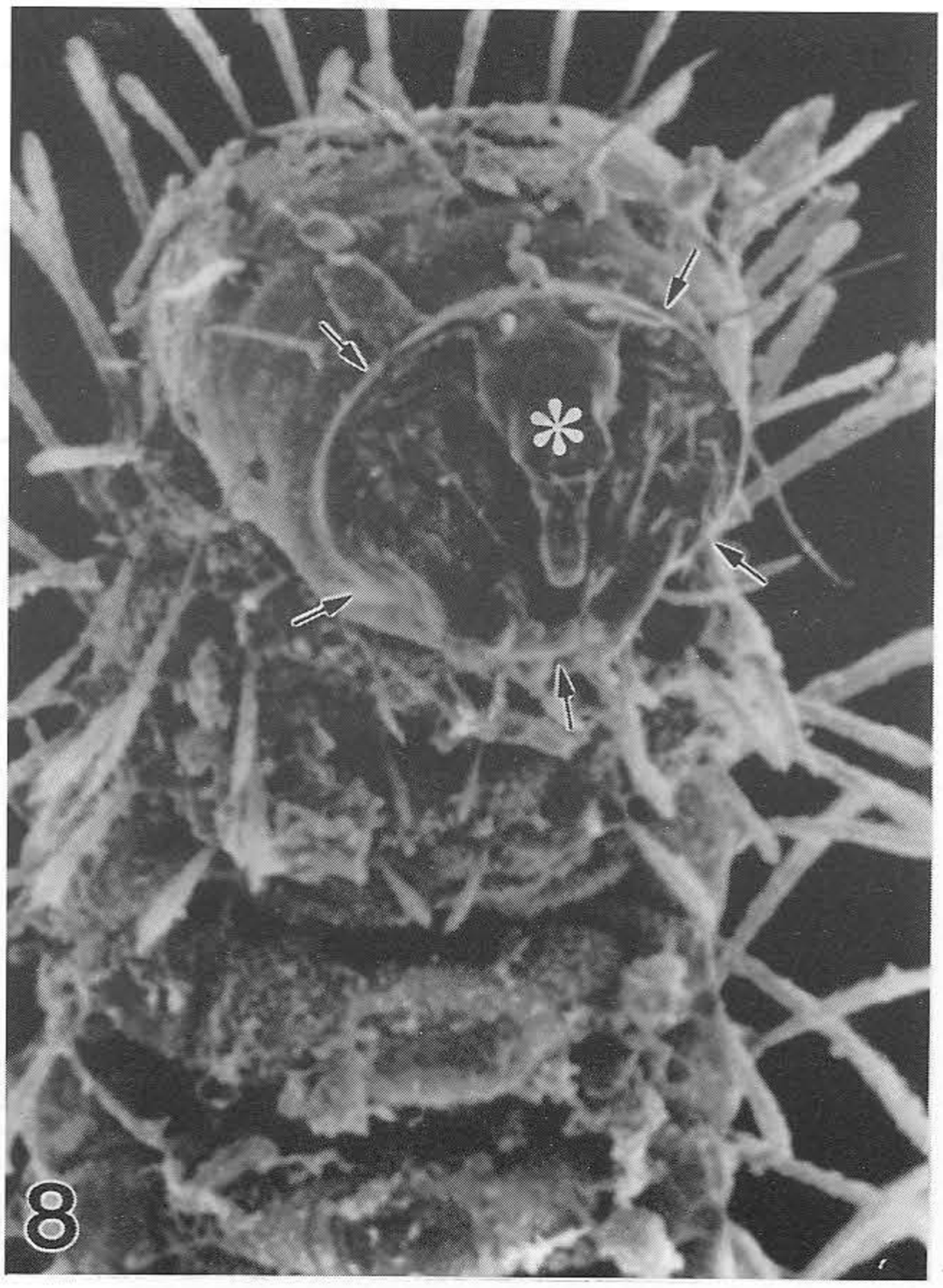

Fig. 8 . Details of the larva head. The anatomical parts of the mouth are distinguished on the frontal part of the head (arrows) showing the labrum (asterisk). X 350

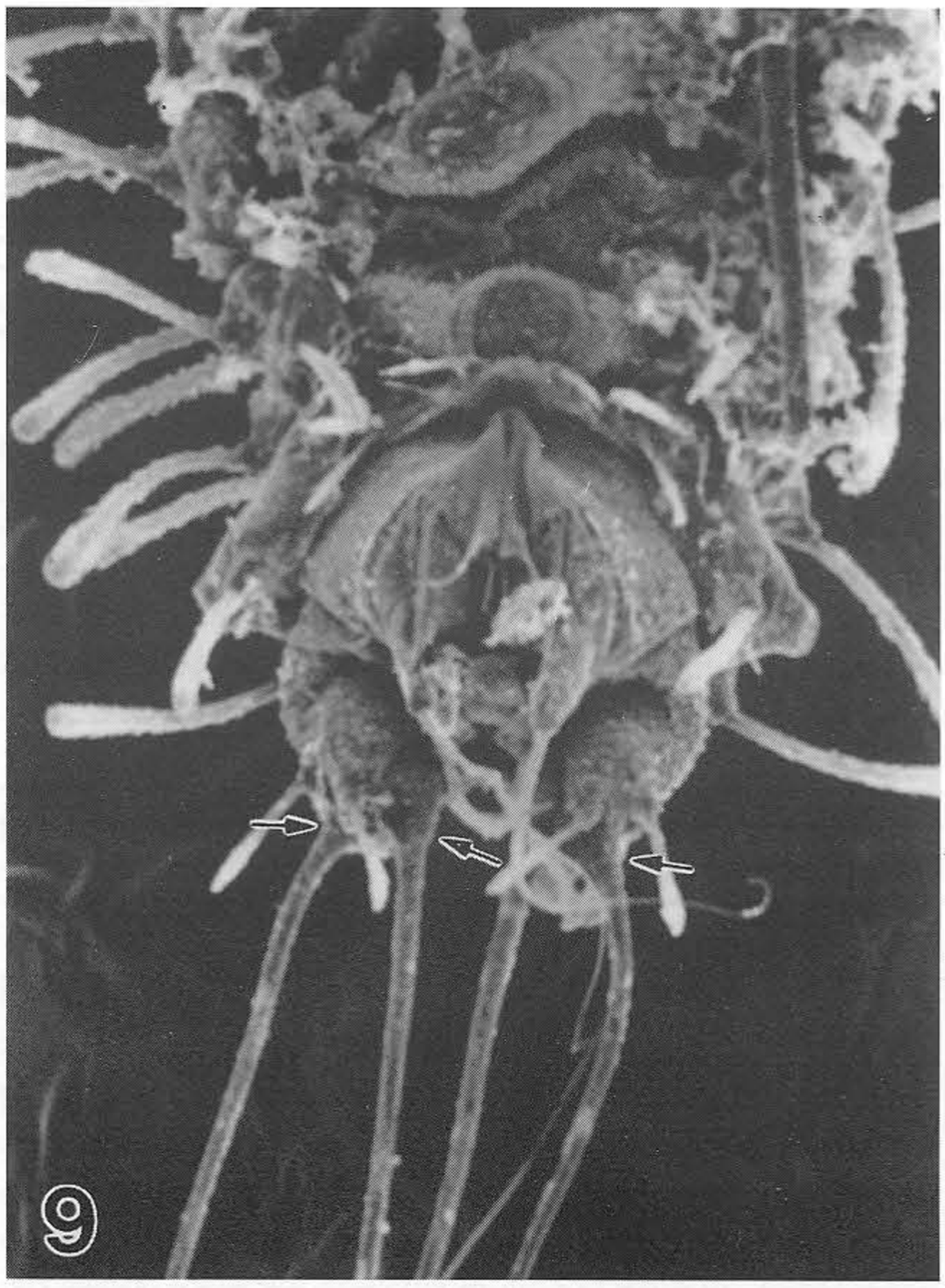

Fig. 9. Details of the larva last segment showing the insertion of the caudal setae. X 350

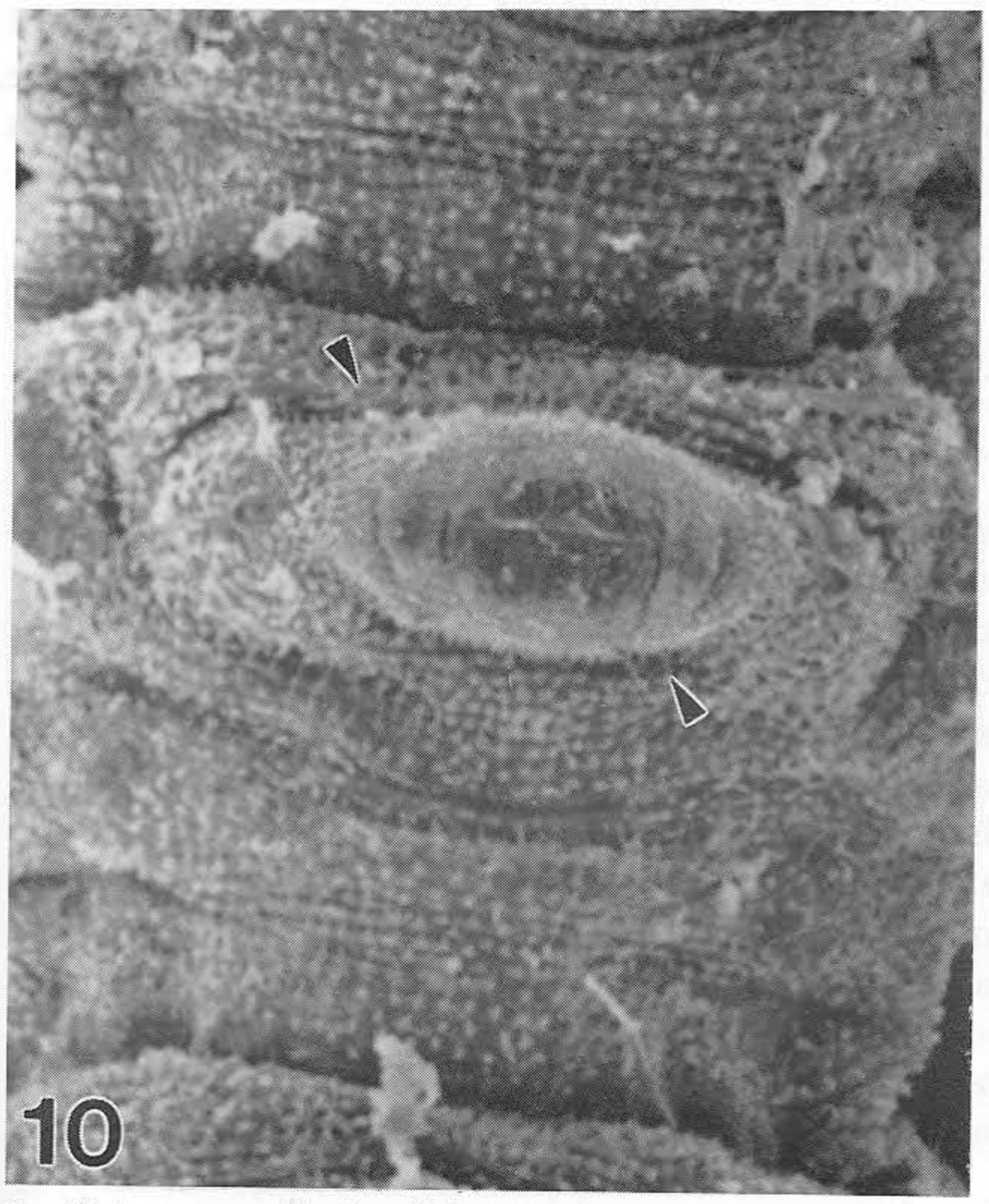

Fig. 10. Large magnification of the ventral part of the larva showing a pseudo-leg (arrowheads). X 600

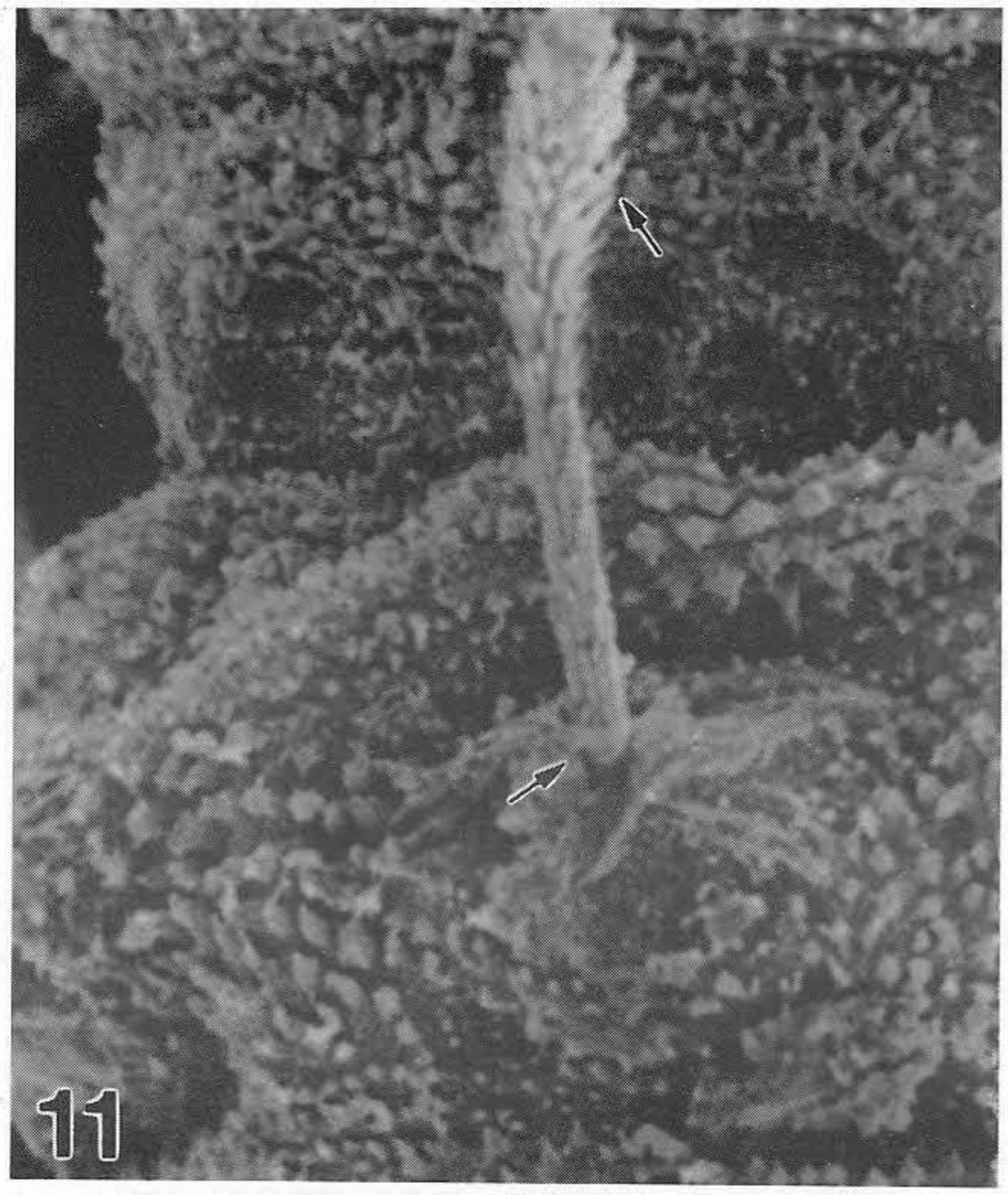

Fig. 11. Large magnification of a larva body showing a seta shape hair (arrowheads). Note that the hair is composed of minute slivers. X 1,200

Lu. longipalpis, as has already been suggested by molecular biology and biochemical studies.

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