Morphological Study of the Larval Spiracular System in Eight *Lutzomyia* Species (Diptera: Psychodidae)

Anna Maria Fausto, M Dora Feliciangeli*/+, Michele Maroli, Massimo Mazzini**

Dipartimento di Scienze Ambientali, Università della Tuscia, Viterbo, Italy *Universidad de Carabobo, Facultad de Ciencias de la Salud, BIOMED, Núcleo Aragua, Maracay, Venezuela **Laboratorio di Parassitologia, Istituto Superiore di Sanità, Roma, Italy

The morphology of the spiracles of fourth instar larva in eight sandfly species were examined by light and scanning electron microscopy. Species studied were: Lutzomyia longipalpis *(Lutz & Neiva)*, L. ovallesi *(Ortiz)*, L. youngi *Feliciangeli & Murillo*, L. evansi *(Nuñez-Tovar)*, L. trinidadensis *(Newstead)*, L. migonei *(França)*, L. absonodonta *Feliciangeli*, and L. venezuelensis *(Floch & Abonnenc).*

In larvae of all eight species both thoracic and abdominal spiracles are located at the top of a globular bulge. Their structure consists of a spiracular plate with a sclerotized central portion and a rose-like peripheral portion. The latter has circularly arranged papillae, separated from each other by elongated septa. Each papilla is longitudinally crossed by a fine cleft dividing it into two identical parts. The taxonomic and adaptative value of spiracular morphology is discussed.

Key words: sandfly - thoracic and abdominal spiracles - amphipneustic larvae - light and scanning electron microscopy

In Insecta, the larval spiracular system assumes a great variety of forms, many of which are clearly adaptative. Despite such as indicative signal, little work has been carried out on the spiracular system in larval stages of different insects (Beckel 1958, Hinton 1967, Berberet & Helms 1972, Khole 1979, Roberts 1981, Nikam & Khole 1989, Principato & Tosti, 1988, 1989). It is generally accepted that the highest number of functional spiracles in existing insects is ten pairs, two of which are thoracic and eight abdominal. On the basis of the number and location of functional spiracles, Keilin (1944) proposed a classification of the larval spiracular system that has been followed until now.

In Diptera, the internal morphology of the tracheal system is rather constant in the various families, while numerous variations are evident in spiracles (Whitten 1955). The structure of spiracles in dipterous larvae varies not only with the species but often with their position on the body (Keilin 1944). Thus, each species may show different kinds of spiracles. Such polymorphism is often intimately connected with the respiratory adaptation of insect larval stages to surrounding conditions. The study of the respiratory adaptations of dipterous larvae to the great variety of external factors revealed forms of convergency of certain structures in species phylogenetically distinct as well as divergency in closely allied groups (Keilin 1944, Whitten 1955).

For only a few of the species of phlebotomine sandflies were the morphological characters of immature stages been studied in the past (Grassi 1907, Saccà 1950, Abonnenc 1956, Abonnenc & Larivière 1957, Trouillet 1976, 1977, 1979). With the current increase in the colonization and rearing of many sandfly species (Killick-Kendrick et al. 1991), there are now opportunities to describe the morphology of eggs, larvae and pupae of the colonized species (Lane & El Sawaf 1986, Killick-Kendrick et al. 1989, Endris et al. 1987, Fausto et al. 1992, 1993, Feliciangeli et al. 1993, Rios & Williams, 1995, Ghosh & Mukhopadhway 1996).

Up to now, the larval spiracles of sandflies have summarily been described for a limited number of *Phlebotomus* species (Abonnenc 1972, Maroli et al. 1992). Moreover, no data are available on their ultrastructure and taxonomic significance.

In the present investigation, the morphology of the larval spiracles in eight neotropical species [*Lutzomyia longipalpis* (Lutz & Neiva 1912), *L. ovallesi* (Ortiz 1952), *L. youngi* Feliciangeli & Murillo 1987, *L. evansi* (Nuñez-Tovar 1924), *L. trinidadensis* (Newstead 1922), *L. migonei* (França 1920), *L. absonodonta* Feliciangeli 1995, and *L.*

This work was supported by the Commission of the European Community, Science and Technology for Developing Countries Program (STD3-TS3*-CT93-0247).
+Corresponding author. Fax: +58 43 71 34 56
Received 20 June 1997
Accepted 27 August 1997

venezuelensis (Floch & Abonnenc 1948)] were examined by light and scanning electron microscopy (SEM). Particular attention was given to a comparison among them and with those of other dipterous larvae in order to point out taxonomic and phylogenetic significance in the subfamily Phlebotominae.

MATERIALS AND METHODS

Fourth instar larvae of *L. longipalpis*, *L. ovallesi*, *L. youngi*, *L. evansi* , *L. trinidadensis*, *L. migonei*, *L. absonodonta*, and *L. venezuelensis* used in the present study were obtained from eggs laid by wild females collected in different habitats in Venezuela and reared at the University of Carabobo, Maracay, following the methods described by Killick-Kendrick et al. (1973).

For light microscopy, adbominal spiracles of larvae were dissected, mounted on slides in mounting medium and directly observed under Axiophot Zeiss light microscope. Portions of larval abdomens were fixed in Bouin's fixative and embedded in paraffin for histological examination. Sections of 7 µm thickness were stained with toluidine bleu and observed under Axiophot Zeiss light microscope.

For SEM, an average of 7 larvae of each species (see Table) were treated with trypsin 0.25% for 5 min and fixed for 2 hr in 4% glutaraldehyde and 5% paraformaldehyde in 0.1 M cacodylate buffer at pH 7.2 (Karnovsky 1965). They were then rinsed overnight in cacodylate buffer, dehydrated in a graded ethanol series, dried by the critical point method using liquid CO_2 in a Balzers CPD 020 apparatus, attached to specimen holders, coated with gold in a Balzers Union MED 010 evaporator and observed in a JEOL JMS 5200 electron microscope.

RESULTS

The fourth instar larva of a sandfly species is amphipneustic, having two pairs of spiracles: the metathoracic pair is situated at the anterior edge of the second thoracic segment and the abdominal one in the posterior corner of the eight abdominal segment (Fig. 1). Although spiracle is smaller, both the thoracic and abdominal spiracles have the same morphological basal plan (Figs 2-5).

In all species studied both thoracic and abdominal spiracles are placed at the top of a globular bulge. Their structure consists of a spiracular plate with a sclerotized central portion and a peripheral portion. The central portion shows chitinous plaques, which vary in number and morphology. The peripheral portion consists of circularly arranged papillae, separated one another by elongated septa (Figs 3, 5). Each papilla is longitudinally crossed by a fine cleft which divides the papilla in two identical parts (Figs 3, 5). The thoracic spiracles always have fewer papillae than the posterior ones. The number of papillae of both thoracic and abdominal spiracles differs from species to species and often between the individuals of the same species.

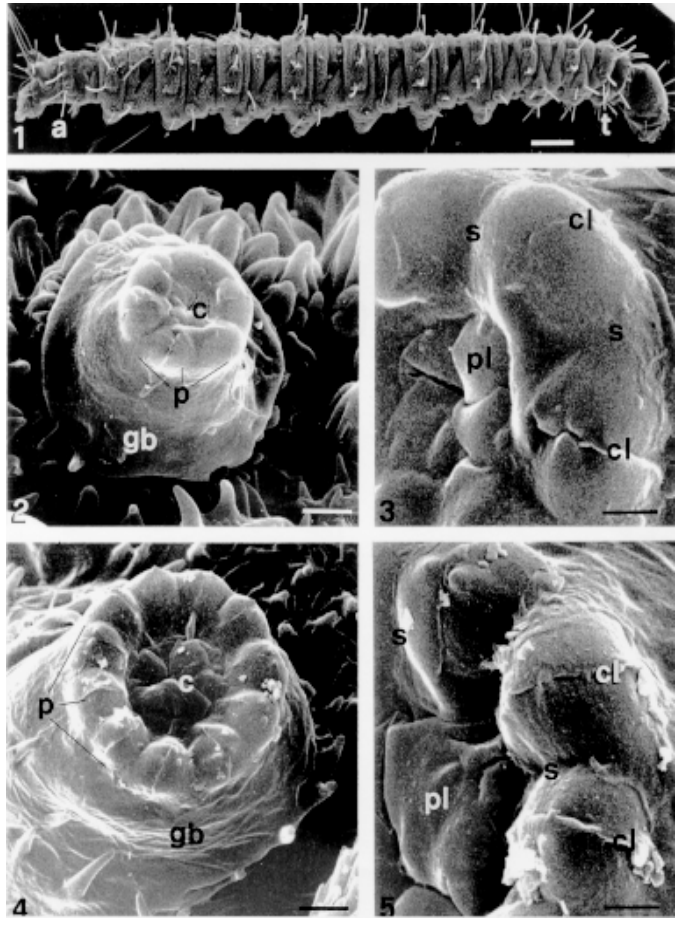
Among the eight species examined, the number of the papillae of the thoracic spiracles varies from six in *L. youngi* and *L. trinidadensis* (Figs 2, 6) to nine in *L. longipalpis* (Fig. 8). Abdominal spiracles have a minimum of 11 papillae in *L. youngi* (Fig. 4) and a maximum of 19 in *L. longipalpis* (Fig. 9). Table shows the number of spiracle papillae (thoracic and abdominal) for each species. Among the species studied, *L. longipalpis* has the largest thoracic and

TABLE

Features and number of papillae observed in the anterior and posterior larval spiracles of eight neotropical phlebotomine sandfly species

Species	No. of larvae studied	Features of the larval spiracles		No. of spiracular papillae ($\bar{x}$)	
		Peripheral area	Central area	T [a]	A [b]
Subgenus *Lutzomyia*					
L. longipalpis	7	Papillae not well defined	No evident plaques	10	18.4
Subgenus *Micropygomyia*					
L. venezuelensis	6	Papillae not well defined	Irregular plaques	7	14.5
L. absonodonta	6	Papillae not well defined	Irregular plaques (4)	7.6	11.6
Species Group *Migonei*					
L. migonei	7	Papillae sharply defined	Triangular plaque (1)	7	12.4
Species Group *Oswaldoi*					
L. trinidadensis	7	Papillae sharply defined	Irregular plaques	6	12
Species Group *Verrucarum*					
L. evansi	7	Papillae sharply defined	Irregular plaques	8.4	12
L. ovallesi	8	Papillae sharply defined	Regular plaques (4)	8	14
L. youngi	9	Papillae sharply defined	Regular plaques (4)	6	10.6

a: thoracic; *b*: abdominal

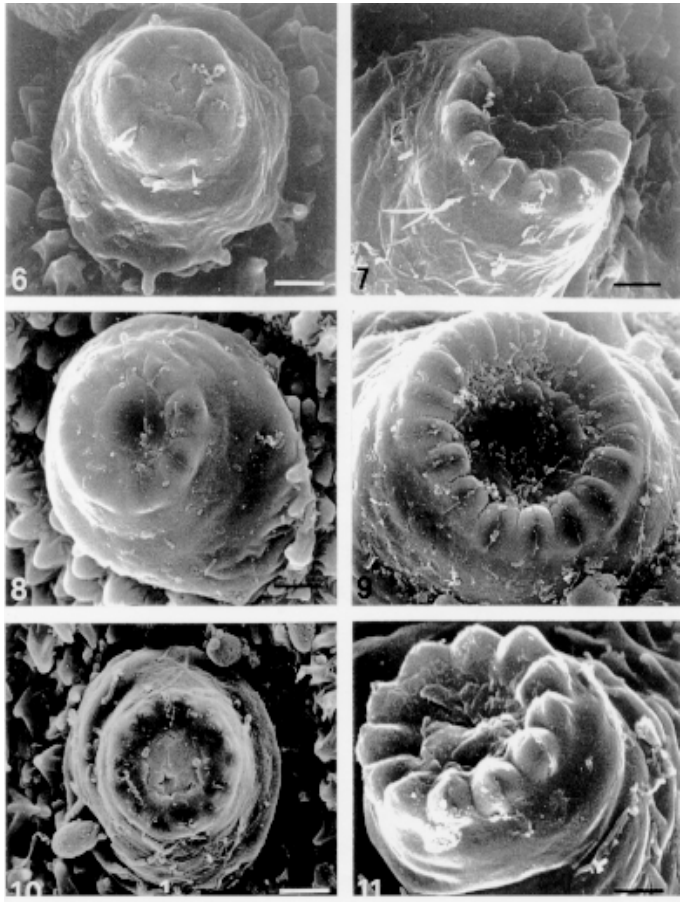


Scanning electron micrographs of fourth larval stage of *Lutzomyia youngi*. Fig. 1: the entire larva shows thoracic (t) and abdominal (a) spiracles. Fig. 2: the thoracic and abdominal spiracles (Fig. 4) are placed at the top of globular bulge (gb). Their structure consists of central (c) and peripheral (p) portions. Fig. 3: a particular of the thoracic and abdominal (Fig. 5) spiracles shows the central portion with chitnous plaques (pl), and the peripheral portion consisting of circularly arranged papillae, separated one another by elongated septa (s). Note the clefts (cl) dividing each papilla in two parts. Fig. 1, bar = 250 mm; Figs 2, 4, bars = 3 mm; Figs 3, 5, bars = 1mm.

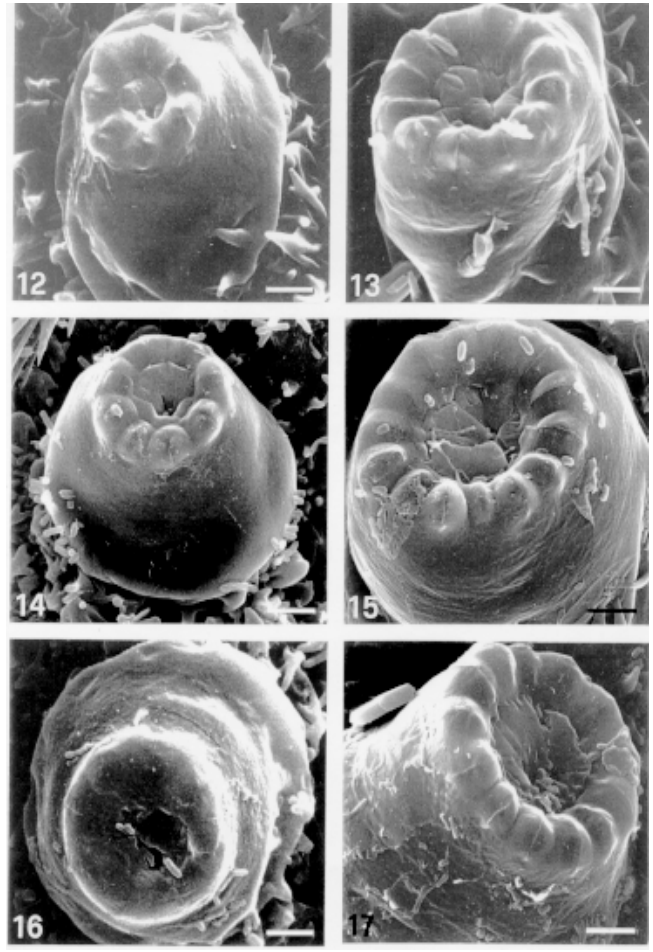
abdominal spiracular structures (Figs 8, 9), and *L. youngi* the smallest (Figs 2-5).

The peripheral portion of both thoracic and abdominal spiracles shows two different features: (i) it appears well defined at the base with papillae sharply distinct one from the other in *L. youngi* (Figs 2-4), *L. trinidadensis* (Figs 6, 7), *L. evansi* (Figs 10, 11), *L. ovallesi* (Figs 14, 15) and *L. migonei* (Figs 18, 19); (ii) it not only continues indefinitely from the basal bulge but also the papillae are slightly separated in *L. longipalpis* (Figs 8, 9), *L. absonodonta* (Figs 12, 13) and *L. venezuelensis* (Figs 16, 17).

Some variations are also evident in the morphology of the central portion of the spiracular plates of the various species. In *L. youngi* (Figs 2-5) and *L. ovallesi* (Figs 14, 15) this structure consists of four similar triangular plaques, with the bases lining the papillae of the peripheral portion and the apices in the centre of the structure. The surface of the triangular plaques are more or less convex, so that depressions are visible among them as well as in the central point where the plaques converge. In *L. trinidadensis* (Figs 6, 7), *L. evansi*



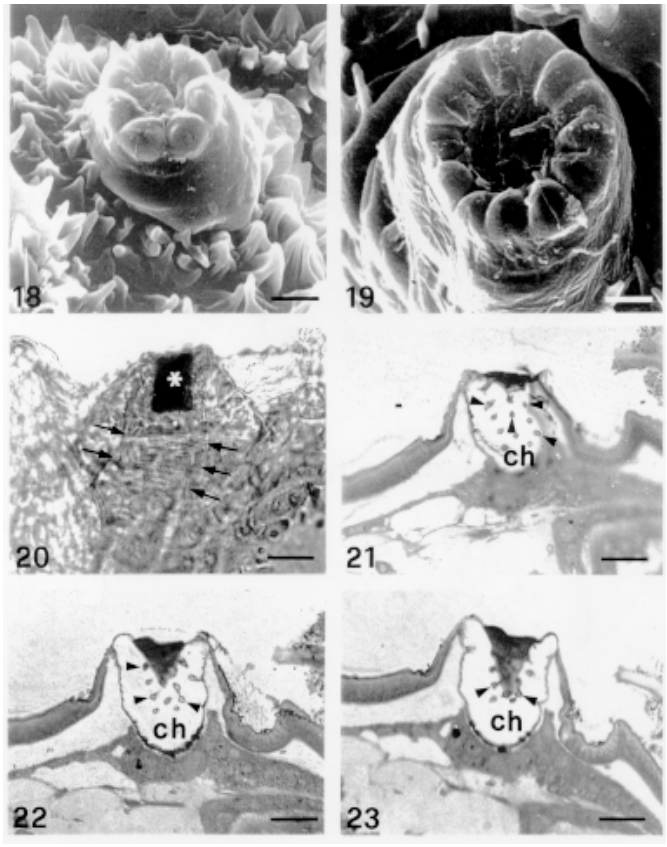
Scanning electron micrograph view of thoracic and abdominal spiracles of *Lutzomyia trinidadensis* (Figs 6, 7); *L. longipalpis* (Figs 8, 9); *L. evansi* (Figs 10, 11). Bars = 3 mm.



Scanning electron micrograph view of thoracic and abdominal spiracles of *Lutzomyia absonodonta* (Figs 12, 13); *L. ovallesi* (Figs 14, 15); *L. venezuelensis* (Figs 16, 17). Bars = 3 mm.

(Figs 10, 11), *L. absonodonta* (Figs 12, 13) and *L. venezuelensis* (Figs 16, 17) the plaques are irregular in shape and not well lined, so that it is difficult to determine their number. They converge towards a point that is in an eccentrical position. In *L. migonei*, the chitinous central portion of the thoracic spiracle consists of only one large triangular plaque (Figs 18, 19). In *L. longipalpis*, the plaques are not-well evidenced and the central portion of the spiracular plate looks like a unique chitinous surface with irregular morphology (Figs 8, 9).

Light microscopy observations of an abdominal spiracle of *L. venezuelensis* show a dark central plug which can be seen through the transparent external chitinous sheet (Fig. 20). The space encircling the plug connects the peripheral region of the external spiracular plate with the tracheal system. In longitudinal cross-sections at different levels of the abdominal spiracle, the central plug consists of electrondense plaques (Figs 21-23). The spiracular plate is covered by a thin chitinous sheet that is continuous with the internal spiracular chamber. The latter, that can be considered a felt chamber, appears to be filled with numerous chitinous projections originating from its wall and directed towards the plug (Figs 21-23).



Scanning electron micrograph view of thoracic and abdominal spiracles of *Lutzomyia migonei* (Figs 18, 19), bars = 3 mm. Light microscopy image of abdominal spiracle in *L. venezuelensis* (Fig. 20); it is evident the dark central plug (asterisk) and the beginning of the tracheal system (arrows), bar = 10 mm. Longitudinal cross sections at different levels of abdominal spiracle of *L. venezuelensis* (Figs 21-23); the internal spiracular chamber (ch) appears to be filled with numerous chitinous projections (arrowheads) originating from its wall and directed towards the plug, bars = 10mm.

DISCUSSION

As reported by previous authors (Keilin 1944, Whitten 1955, Abonnenc 1972), the larvae of all Psychodidae are amphipneustic, having a pair of thoracic and abdominal spiracles. It is now generally accepted that the polypneustic system, with 8-10 pairs of functional spiracles, characterizes the terrestrial mode of life.

The oligopneustic (which comprises the amphipneustic type) and apneustic larvae, with 0-4 pairs of functional spiracles, may be a subsequent adaptation to a subemerged life in a fluid or semi-fluid medium. This condition, derived from the primitive terrestrial polypneustic form, is considered more specialized (Keilin 1944).

The lack of an intermediate system between the poly and oligopneustic systems is due to the fact that the first seven pairs of abdominal spiracles are either all present or all absent. The simultaneous closure of eight pairs of spiracles (one thoracic and seven abdominal) is probably due to the partial immersion of the larva in a liquid medium, a factor that acts more or less uniformly on all segments of the body except the two terminal ones which are in a position to bring their spiracles in contact with the air. In different species, these spiracles are modified for adaptation to a wide diversity of larval habits.

Among Psychodidae, the aquatic larvae of Psychodinae have the post-abdominal spiracles opening at the end of a respiratory siphon, as an adaptation to the aquatic mode of life, while the present investigation shows that larvae of Phlebotominae which live in a decomposed organic matter are completely devoid of such a syphon. In sandflies, the spiracles emerge on the top of globular structures and project from the surface of the larval body for their localization. This localization is probably an adaptation to life in the decomposed organic matter, which favours the contact between the spiracles and the air.

In other dipteran species living in similar habitats, for example in the stratiomyid *Metopina rubiceps* and *Microchrysa polita*, the postabdominal spiracles are found within a pneumatic sac which opens up to the exterior by means of a transverse slit (Keilin 1944). The larvae having a pneumatic sac are able to survive by having only intermittent contact with the air whose admission into the sac is controlled by dilatatory muscles.

We have no evidence on the mechanism controlling air admission in sandfly larval spiracles. Light observations of the internal structural arrangement do not show the presence of muscles or other structures involved in regulatory function. Further studies need to understand the fine organization of the spiracular apparatus and clarify the modality of its regulation.

An evident aspect of the sandfly larval spiracular system is the different size between the thoracic and abdominal spiracles. In the dipteran larval spiracular system, postabdominal spiracles are generally the most developed. In some cases, the thoracic spiracles differ from the postabdominal not only in structure but also in function. In the dipteran *Gasterophilus* larvae, the thoracic spiracles have an internal occlusion that prevents the entry of extraneous material and help the larva to survive in the gastro-intestinal tract (Principato & Tosti 1988). Thus *Gasterophilus* larvae, appearing to be morphologically amphipneustic, are functionally metapneustic, having thoracic spiracles with an opening similar to those of the abdominal spiracles.

Among Psychodinae, some forms have been identified as having a metapneustic respiratory system. Our observations show that the posterior spiracles of fourth stage sandfly larvae appear to be almost functioning. They are characterized by a large internal spiracular chamber that the air probably reaches by passing through the longitudinal clefts of the peripheral spiracular plate. The sclerotized plug might be formed by the contraction and hardening of the chitin surrounding the ecdysial opening through which the tracheospiracular system of the previous larval stage is expelled. The opening thus becomes obliterated. This spiracle is considered Type II. In fact, the spiracles in dipterous larvae can be separated into three main types on the basis of the process of moulting between two successive larval stages (Keilin 1944). Type I is characterized by spiracles in which the ecdysial opening becomes the spiracular opening of successive stages. In Type II, the ecdysial opening is obliterated and a spiracular plate with clefts papillae are formed around the ecdysial scar which is more or less central. Type III is similar to Type II except that the spiracular plate lies outside the ecdysial scar and does not surround it. However, further studies on the development of the spiracular system throughout the different sandfly larval stages need to confirm this suggestion.

It is possible that in the sandfly species studied the formation of the central chitinous scar plug is the result of various processes which determine four similar triangular plaques in *L. youngi*, *L. absonodonta* and *L. ovallesi*, some irregular plaques in *L. evansi*, *L. trinidadensis* and *L. venezuelensis*, only one plaque in *L. migonei* and *L. longipalpis*. In the different species, the morphology of the spiracles also varies in the number of papillae and in their aspect as shown in Table. Because of the small number of species studied, it is not possible at present, to establish any relationship between the features of the fourth instar larvae spiracles of phlebotomine sandfly species and their taxonomic status. From these first observations it seems that the appearance of the peripheral portion of the spiracles might be a constant character among species of the same subgenus or species-group. Additionally, the combined number of papillae of the thoracic and abdominal spiracles might help in distinguishing species of the same group, which also show a similar structure of the central portion of the spiracles (e.g. *L. ovallesi* from *L. youngi*) (Table). However, more information is needed on a greater number of species of the same taxa to reach such a conclusion. On the other hand, the adaptative significance of the morphological variation in the spiracular system of different species of phlebotomine sandfly four instar larvae is, at the moment, hard to understand due to the lack of information about their microhabitats.

REFERENCES

Abonnenc E 1956. Sur la morphologie de *Phlebotomus freetownensis* var. *magnus* Sinton, 1932 et de *Phlebotomus freetownensis* var. *sudanicus*, Theodor, 1933, d'après des individus provenat d'élevage. *Arch Inst Pasteur Algér 34*: 388-399.

Abonnenc E 1972. Les Phlébotomes de la région éthiopienne (Diptera, Psychodidae). *Mem ORSTOM 55*: 1-288.

Abonnenc E, Larivière M 1957. Les formes larvaires de quelques Phlèbotomes des règions Mèditerranèenne et Ethiopienne. *Arch Inst Pasteur Alger 35*: 391-403.

Beckel WE 1958. The morphology, histology and physiology of the spiracular regulatory apparatus of *Hyalophora cecropia* (L.) (Lepidoptera). *Proc 10th Intern Congr Entomol 2*: 87-115.

Berberet RC, Helmes TJ 1972. Comparative anatomy and histology of selected systems in larval and adult *Phyllophaga anxia* (Coleoptera: Scarabaeidae). *Annals Entomol Soc Amer 65*: 1026-1053.

Endris RG, Young DG, Perkins PV 1987. Ultrastructural comparison of egg surface morphology of five *Lutzomyia* species (Diptera: Psychodidae). *J Med Entomol 24*: 412-415.

Fausto AM, Maroli M, Mazzini M 1992. Ootaxonomy and eggshell structure of *Phlebotomus* sandflies. *Med Vet Entomol 6*: 201-208.

Fausto AM, Maroli M, Mazzini, M 1993. Scanning electron microscopical study of the eggshell of three species of *Sergentomyia* (Diptera, Psychodidae). *Insect Sci Appl 14:* 483-488.

Feliciangeli MD, Castejon OC, Limongi J 1993. Egg surface ultrastructure of eight New World Phlebotomine sand fly species (Diptera: Psychodidae). *J Med Entomol 30*: 651-656.

Ghosh KN, Mukhopadhway JA 1996. A comparison of chorionic sculpturing of four Indian phlebotomine sandflies (Diptera: Psychodidae) by scanning electron microscopy. *Parasitology 3*: 61-68.

Grassi B 1907. Ricerche sui flebotomi. *Mem Soc Ital Sci 14*: 353- 394.

Hinton HE 1967. The structure and function of the spiracular gill of the fly *Taphrophila vitripennis*. *Proc Roy Soc (London) Ser B 147*: 90-120.

Karnovsky MJ 1965. A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. *J Cell Biol 27*: 137A-138A.

Keilin D 1944. Respiratory systems and adaptations in the larvae and pupae of Diptera. *Parasitology 36*: 1-66.

Khole V 1979. Spiracular development in the blowfly larvae (Calliphoridae: Diptera) *Biovigjanam 5*: 43-52

Killick-Kendrick R, Leaney AL, Ready PD 1973. The establishment, maintenance and productivity of a laboratory colony of *Lutzomyia longipalpis* (Diptera: Psychodidae). *J Med Entomol 13*: 429-440.

Killick-Kendrick R, Killick-Kendrick M, Leger N, Pesson B, Madulo-Leblond G 1989. Absence of outer caudal setae on all larval instars of *Phlebotomus tobbi* from the Ionian Greek islands. *Med Vet Entomol 3*: 131-135.

Killick-Kendrick R, Maroli M, Killick-Kendrick M 1991. Bibliography on the colonization of phlebotomine sandflies. *Parassitologia 35*: 321-333.

Lane RP, El Sawaf B 1986. The immature stages of *Phlebotomus langeroni* (Diptera: Psychodidae). *J Med Entomol 23*: 263-268.

Maroli M Fausto AM, Mazzini M 1992. Morfologia degli stadi larvali dei flebotomi (Diptera: Psychodidae). 1. Gli stigmi respiratori di *Phlebotomus papatasi*, *P. perniciosus* e *P. perfiliewi*. *Parassitologia 34 (Suppl. 1)*: 221-222.

Nikam TB, Khole VV 1989. *Insect Spiracular Systems*, J Wiles & Sons, Halsted Press, Chichester, 135 pp.

Principato M, Tosti M 1988. Scanning electron microscope observations on the anterior thoracic and posterior post-abdominal spiracles of *Gasterophilus* larvae (Diptera: Gasterophilidae). *Int J Parasitol 18*: 191-196.

Principato M, Tosti M 1989. Le morphologie interne des stigmates protoraciques chez le troisième stade larvaire de *Gasterophilus intestinalis* (Diptera: Gasterophilidae) en microscopie électonique a balayage et a transmission. *Ann Parasitol Hum Comp 64*: 143-149.

Rios Leite AC, Williams P 1995. Morphology and chetotaxy of the fourth instar larva of *Lutzomyia longipalpis* (Diptera: Phlebotomidae) based on scanning electron microscope. Talleres No. 4. Second International Symposium on Phlebotomine Sandflies, Mérida, Venezuela, p. 88.

Roberts B 1981. Development of the prothoracic spiracles in larvae of *Sarcophaga bullata* (Sarcophagidae: Diptera). *Aust J Zool 29*: 199-204.

Saccà G 1950. Stadi preimaginali di *Phlebotomus perfiliewi* Parrot, *P. papatasi* Scop., *P. perniciosus* Newstead (Diptera: Psychodidae). *Rend Ist Super Sanità 13*: 680-688.

Trouillet J 1976. *Sergentomyia (Grassomyia) ghesquierei* Parrot 1929 (Diptera, Phlebotomidae). Etude morphologique des stades pré-imaginaux et notes bio-écologiques. *Cah ORSTOM Ser Entomol Med Parasit 14*: 347-356.

Trouillet J 1977. *Sergentomyia (Sergentomyia) bedfordi firmatus* Parrot et Malbrant, 1945 et *Sergentomyia (Sergentomyia) bedfordi medius* Kirk et Lewis, 1950 (Diptera, Phlebotomidae). Etude morphologique des stades pré-imaginaux et notes bio-écologiques. *Ann Parasitol Hum Comp 52*: 35-52.

Trouillet J 1979. *Sergentomyia (Rondanomyia) ingrami* Newstead, 1914, *Sergentomyia (Rondanomyia) dureni* Parrot, 1934 et *Sergentomyia (Sergentomyia) hamoni* Abonnenc, 1958 (Diptera, Phlebotomidae). Etude morphologique des stades pré-imaginaux et notes bio-écologiques. *Ann Parasitol Hum Comp 54*: 353-373.

Whitten JM 1955. A comparative morphological study of the tracheal system in larval diptera. Part I. *Q J Microsc Sci 96*: 257-278.