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Short Communication

Ultrastructural characterization of the pre-adult stages of *Drosophila willistoni* species group (Diptera, Drosophilidae)

Rebeca Zanini[1,2,5], Maríndia Deprá[1,2,3,6] and Vera Lúcia da Silva Valente[1,2,4,7]

[1]Programa de Pós-Graduação em Biologia Animal (PPGBAN), Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil; [2]Laboratório de *Drosophila*, Instituto de Biociências, Departamento de Genética, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil; [3]Departamento de Ecologia, Zoologia e Genética, Instituto de Biologia, Universidade Federal de Pelotas (UFPel), Pelotas, RS, Brazil; [4]Programa de Pós-Graduação em Genética e Biologia Molecular (PPGBM), Instituto de Biociências, Universidade Federal do Rio Grande do Sul (UFRGS), Porto Alegre, RS, Brazil.

ABSTRACT

Drosophila willistoni group is a Neotropical model for evolutionary studies and has been explored since the latter half of the past century. The *willistoni* group encompasses 24 species divided into *alagitans*, *bocainensis* and *willistoni* subgroups. This group has a very interesting evolutionary and taxonomic history, characterized by the different speciation levels of its species – some seem to be in the process of speciation whereas others already have completed it. Despite the amount of studies on this group, there is a lack of morphological studies, especially concerning the pre-adult stages of development. Thus, the objective of this study was to characterize the external morphology of the eggs, larvae and pupae of the *Drosophila willistoni* species group based on Scanning Electron Microscopy. We verified that the morphology of these immature stages are very similar between the analyzed species. The respiratory filaments of the eggs, however, present variation even among the semispecies of the *D. paulistorum* complex. This morphological similarity is compatible with the relatively recent process of speciation and the pattern of substrate exploration.

[5]Email id: rebeca.zanini@gmail.com
[6]Email id: marindiadepra@gmail.com
[7]Email id: vera.valente@pq.cnpq.br

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INTRODUCTION

The *Drosophila willistoni* species group (Diptera, Drosophilidae) is a Neotropical member of the *Sophophora* subgenus [1, 2]. This group includes, in addition to *Drosophila willistoni* Sturtevant 1916, another 23 species divided into *alagitans, bocainensis* and *willistoni* subgroups [3]. The *willistoni* group is known for its complex evolutionary history, characterized by the presence of distinct taxonomic levels – species, subspecies and semispecies [4, 5]. Studies analyzing the morphological characteristics of early developmental stage are still scarce. The study conducted by Kambysellis [6] is one of the main references in the characterization of the eggshell of Drosophilidae. In this study, the author compared the chorionic morphology of several Drosophilidae species, including *D. willistoni* and *D. paulistorum* Dobzhansky and Pavan 1949, and attempted to infer phylogenetic relationships based on eggshell characters. Markow *et al.* [7] presented a comparative study regarding the egg size and time of embryonary development of *D. willistoni* and another species with fully sequenced genome.

Okada [8] characterized eggs, larvae and pupae of several genus and species of Drosophilidae and

created an identification key based on the characters of pre-adults.

The early stages of development of some species of this group were briefly characterized by Dobzhansky [9], who superficially described the external morphology of eggs and pupae of *D. equinoxialis* Dobzhansky 1946. Burla *et al.* [10] characterized eggs and pupae of *D. tropicalis* Burla and Cunha 1949 and *D. paulistorum*. Kastritsis and Dobzhansky [11] also described the general shape of eggs and pupae of *D. pavlovskiana* Kastritsis and Dobzhansky 1966. Dobzhansky *et al.* [12] highlighted the similarity between the eggs of *D. insularis* Dobzhansky 1957 and *D. willistoni.*

Several studies have aimed to unveil the formation routes for chorionic appendixes in *Drosophila* eggs, as well as the pathways that result in the patterns of cuticular denticles and trichomes in larvae. Even though several studies dealt with immature stages of *Drosophila*, only a few studied their external morphology. Therefore, the aim of this study was to comparatively describe the external morphology of eggs, larvae and pupae of species in the *willistoni* group, based on scanning electron microscope (SEM) analysis which could later result in evolutive studies.

MATERIALS AND METHODS

Fly stocks

The stocks were reared in a cornmeal medium [13], at constant temperature and humidity (17 ± 1 ºC; 60% relative humidity), with controlled photoperiod. All species and strains used in this study are listed in table 1.

At least 50 eggs and 10 3rd instar larvae and pupae of each species were observed. Two strains of *D. paulistorum* Andean-Brazilian and five strains of *D. willistoni* from different localities were analyzed in order to verify intraspecific variation. Only one strain of each remaining species was analyzed.

Collecting eggs, larvae and pupae

For the egg collection, flies were transferred to vials containing oviposition medium [14], where they were kept for 3 hours. After this period, eggs were manually collected with a histological needle and stored at 4 °C for 7 days in a fixative solution (12% Glutaraldehyde, 50% Phosphate Buffer, pH 7.4 and 38% distilled water).

Larval and pupal stages were obtained directly from the stock, after transferring the adults to another vial. Larvae were 3rd instar (approximately seven days after fertilization) and pupae 8-12 days after fertilization.

SEM preparation and visualization

Previously fixated eggs were washed thrice in a 1:1 Phosphate Buffer (pH 7.4) and distilled water solution, with 30 minutes for each wash. Next, eggs were dehydrated with acetone washes in the following concentrations: 30%, 50%, 70%, 90% (2X) and 100% (2X), for 10 minutes each. Finally, the eggs were critical point dried in a BALZERS CPD030 using CO_2 and acetone 100% as a transition fluid.

The larvae and pupae were not fixated nor critical point dried. Eggs, larvae and pupae were mounted in stubs with carbon tape and metalized with gold in a BALZERS SCD050 sputter coater. Visualization and image capture were made in a JEOL JSM6060 scanning electron microscope, in the Centro de Microscopia Eletrônica - UFRGS.

Images were processed using Adobe Photoshop CC 2015.

The terminology used in this study followed Kambysellis [6] (eggs), Wipfler *et al.* [15] (larvae) and Okada [8] (pupae).

RESULTS AND DISCUSSION

Eggs

The eggs of analyzed species of the *willistoni* group are whitish oblong structures, approximately 0.5 mm long, and covered with hexagonal follicular imprints (Figures 1A-B). The posterior area of the egg (posterior pole) presents follicular cells modified in pores (Figures 1C-D). The anterior pole presents two respiratory filaments, which are shorter than total egg length, distally expanded, and projecting from the anterior dorsal surface (Figure 1A). The shape of these filaments is subtly different between the analyzed species (Figures 2A-L). The number of respiratory filaments was previously reported for some of the analyzed species [6, 9, 10, 11]. According to Kambysellis [6], the number of respiratory filaments varies among the genus, but tends to be more consistent among each subgenus. The author also stated that all the species of *Sophophora* subgenus have just one pair of equally sized filaments. The presence of two respiratory filaments is pointed out by Okada [8] as a

Table 1. Species and strains of *Drosophila* used in this study.

Subgroup	Species	Semispecies	Locality	Collected by
willistoni	*D. equinoxialis*		Apazapán, Mexico	Lee Ehrmann and Yong Kyu Kim
	D. insularis		St. Lucia, Lesser Antilles	Jeffrey R. Powell
	D. paulistorum	Andino Brasileira	Florianópolis, Brazil	Marco Gottschalk
		Andino Brasileira	Ribeirão Preto, Brazil	Vera L. S. Valente
		Amazônica	Belém do Pará, Brazil	Lee Ehrmann and Yong Kyu Kim
		Centroamericana	Lancetilla, Honduras	Lee Ehrmann and Yong Kyu Kim
		Interior	Llanos, Colombia	Lee Ehrmann and Yong Kyu Kim
		Orinocana	Georgetown, Guyana	Lee Ehrmann and Yong Kyu Kim
		Transicional	Santa Marta, Colombia	Lee Ehrmann and Yong Kyu Kim
	D. tropicalis		San Salvador, El Salvador	Tucson Stock Center
	D. willistoni		Salvador, Brazil (1)	Helga Winge and Antonio Cordeiro
			Guadalupe, Caribbean (2)	Tucson Stock Center
			Coronilla, Uruguay (3)	Beatriz Goñi
			Apazapán, Mexico	Margaret Kidwell
			Ribeirão Preto, Brazil	Cláudia Rohde
bocainensis	*D. capricorni*		Porto Alegre, Brazil	Maríndia Deprá and Brenda G. Alexandre
	D. fumipennis		Joinville, Brazil	Carolina F. Garcia, Hermes J. Schmitz and Juliana Cordeiro
	D. nebulosa		Porto Alegre, Brazil	Carolina F. Garcia
	D. sucinea		Mexico	Tucson Stock Center

diagnostic character of the eggs of *Sophophora* subgenus.

The presence, number and size of respiratory filaments are directly linked to the type of substrate used by each species as an oviposition site [6]. The species of *willistoni* subgroup usually oviposit in fallen and rotten fruits, leaving the respiratory filaments and eventually the micropyle in open air. The distal expansion observed in the respiratory filaments of the eggs of this group (Figures 1A, 2A-L) seems to be befitting with the oviposition behavior, since it increases the available gas exchange surface and aids the egg to anchor to the substrate.

The operculum and micropyle are prominent chorionic structures and are located in the anterior region of the egg. The operculum is the chorionic region surrounded by the base of respiratory filaments, where the larvae emerge. The operculum presents a reticulated surface, which became more globular as the time approaches for the larvae to emerge (Figure 1F). The micropyle is a chorionic structure where the spermatozoa penetrate the egg. This structure is situated in the anterior region of the operculum and is not covered with follicular imprints (Figures 1E, 1F). These structures do not show variation between the analyzed species.

Kambysellis [6] reported differences in the follicular imprints: in some species they are tall and lacy and in other species, they are short and narrow. The follicular imprints of *D. willistoni* species group are short and narrow and do not exhibit differences between the species.

Larvae

Third instar larvae of *willistoni* group reach 3-4 mm in length (Figure 3A) and the cuticle shows little sclerotization. The body is composed of a pseudocephalon, three thoracic segments and eight abdominal segments (Figure 3A). The thoracic and abdominal segments do not present legs or protolegs (Figure 3A).

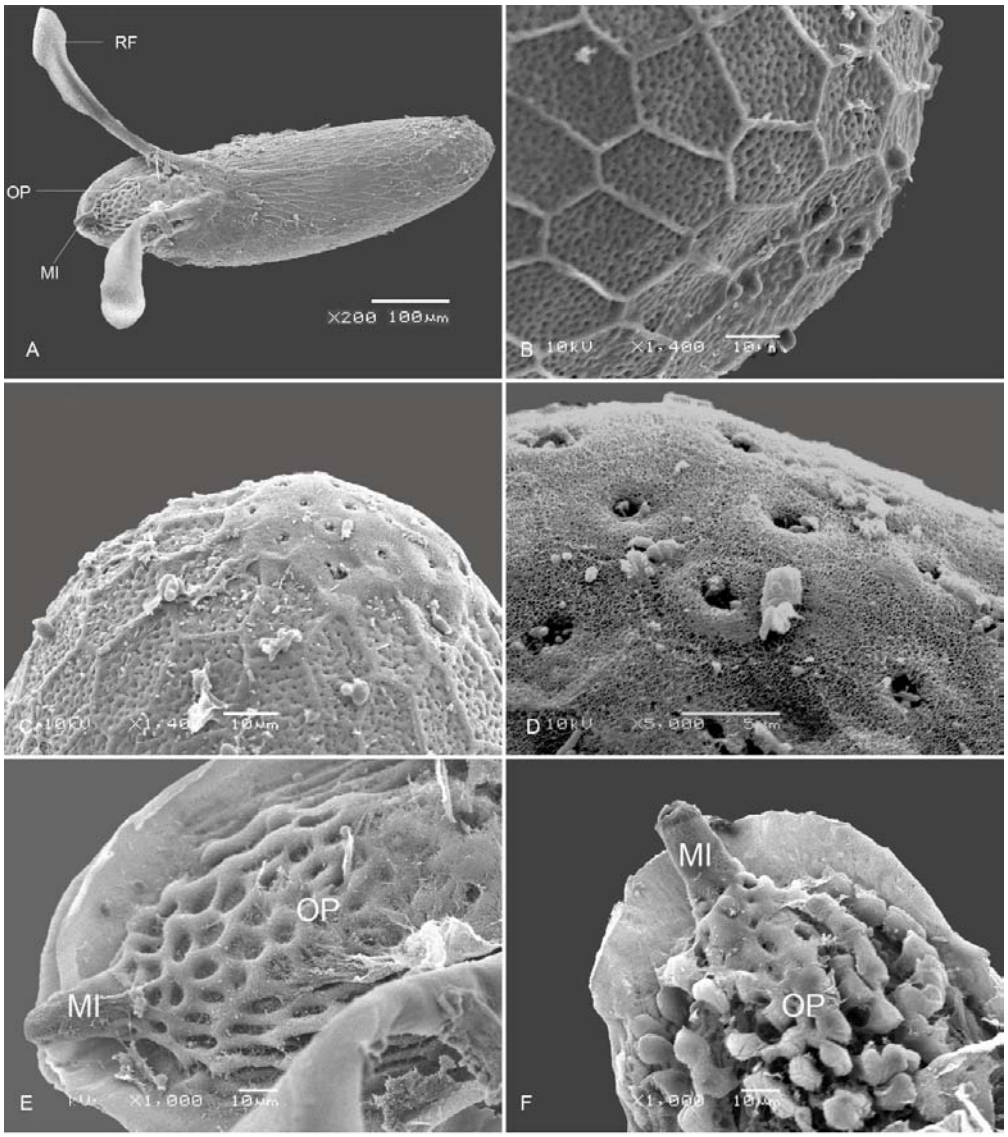

Figure 1. Scanning electron microscopy of the eggs of the *Drosophila willistoni* species group. A. Egg in dorsal view (*D. willistoni*), B. Follicular imprints (*D. tropicalis*), C. Posterior pole (*D. paulistorum* Centroamerican), D. Aeropyles (*D. paulistorum* Centroamerican) and E-F Operculum and micropyle (*D. willistoni* and *D. tropicalis*, respectively).
MI, Micropyle; RF, Respiratory filaments; OP, Operculum.

The cephalic capsule is reduced and internalized (Figures 3A and 3C). The pseudocephalon region presents a pair of maxillary sensorial organs and a pair of antennal organs (Figure 3C). The antennal organs are globular structures and are located between the pair of sensorial organs. The sensorial organs are composed of two sets of sensillae, surrounded by a ring shaped cuticular salience (Figure 3C). The larvae present a pair of sickle-shaped, serrated, heavily sclerotized oral hooks in the ventral region (Figure 3B). The larvae of *willistoni* group present well-developed oral hooks, compared to those of *D. melanogaster*, shown in Wipfler *et al.* [15]. In the oral hook area, we can also observe the cirri, organized in 6-8 parallel rows (Figures 3B, 3C, 3H).

The thoracic segments and the seven first abdominal segments are very similar; each segment present a

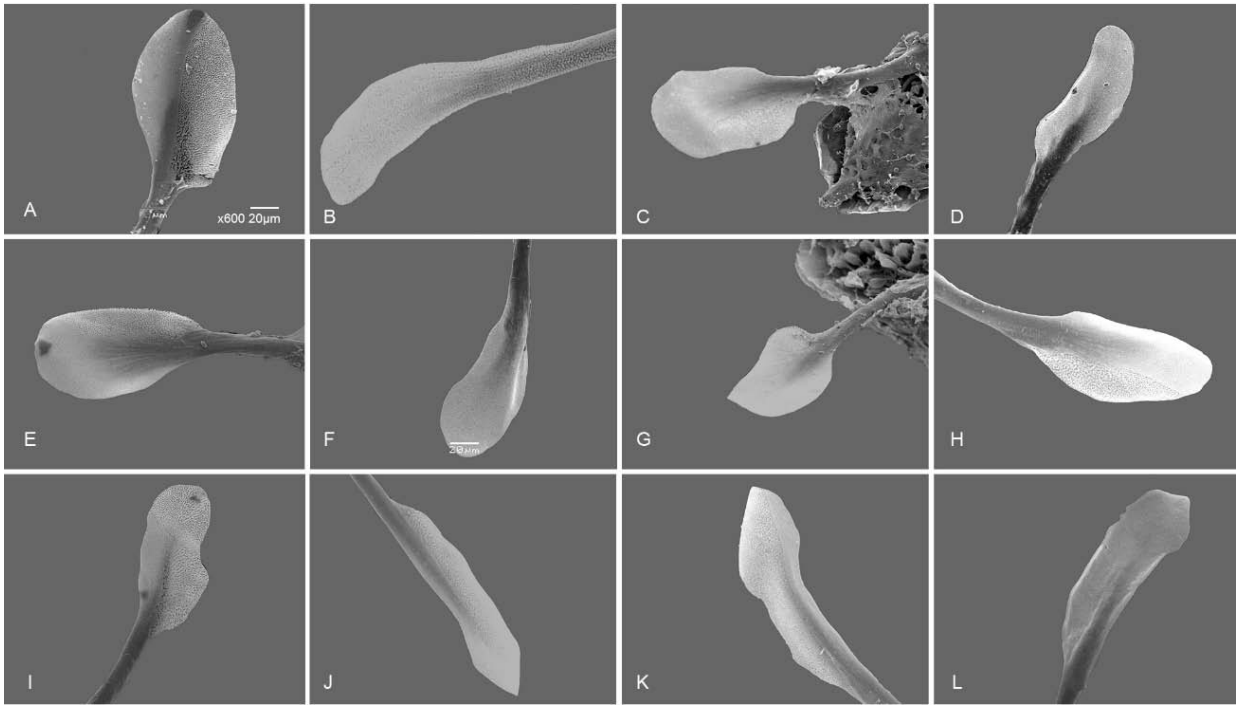

Figure 2. Scanning electron microscopy of the respiratory filaments of the eggs of *Drosophila willistoni* species group. A. *D. equinoxialis*, B. *D. nebulosi*, C. *D. paulistorum* Amazonian, D. *D. paulistorum* Andean-Brazilian, E. *D. paulistorum* Centroamerican, F. *D. paulistorum* Interior, G. *D. paulistorum* Orinocan, H. *D. paulistorum* Transitional, I. *D. tropicalis*, J. *D. willistoni* (1), K. *D. willistoni* (2) and L. *D. willistoni* (3).

transversal band composed of several irregular stripes of cuticular denticles or spines (Figure 3G). These transversal bands, in *willistoni* group, exhibit triangular cuticular denticles and spines and do not present trichomas (Figure 3G), as can be observed in some species of *melanogaster* group [16]. This pattern found in *willistoni* group is similar to those of *D. sechellia* [16] and do not present visible differentiation between the analyzed species, contrasting with *melanogaster* subgroup, in which patterns are species-specific.

The last abdominal segment (A8), the anal segment, is distinctly different from the other segments. This segment is covered with microthrichia, except in the anal organs and in the spiracular tubes (Figures 3A, 3D-F). Anal organs or anal pads are oblong oval structures, with a transversal furrow in the central area (Figures 3A, 3D-F). The anus is located between the anal organs, both of which are in the ventral face of the larvae (Figures 3A, 3D-F).

The abdominal spiracle is formed by spiracular tubes, which are two cylindrical structures connected to the anal segment through the peritreme and are surrounded by six pairs of anal papillae (Figures 3A, 3D-F). Peritreme and spiracular tubes can be everted (Figure 3D) or retracted (Figure 3F). The spiracular tubes present a spiracular scar and have spiracular openings, located laterally to the scar (Figure 3E). Two pairs of anal papillae are adjacent to anal organs and the remaining pairs surround the spiracles (Figures 3C and 3E).

Pupae

The pupae of *willistoni* group are approximately 2.5 mm in length. The coloration varies from light brown, at the beginning of pupal stage, to dark brown at the end. Pupae are elliptic shaped, slightly dorsal and ventrally convex in the anterior region (Figures 4A and 4C). The pupae also present bands of denticles and spines alternated with bands without cuticular ornamentations (Figures 4A, 4C, 4F, 4H).

The species in *willistoni* group present a pair of anterior spiracles, composed of eight ramifications covered with tiny spines (Figures 4D and 4G). According to Okada [8], the number of ramifications of the anterior spiracles is the main distinguishing

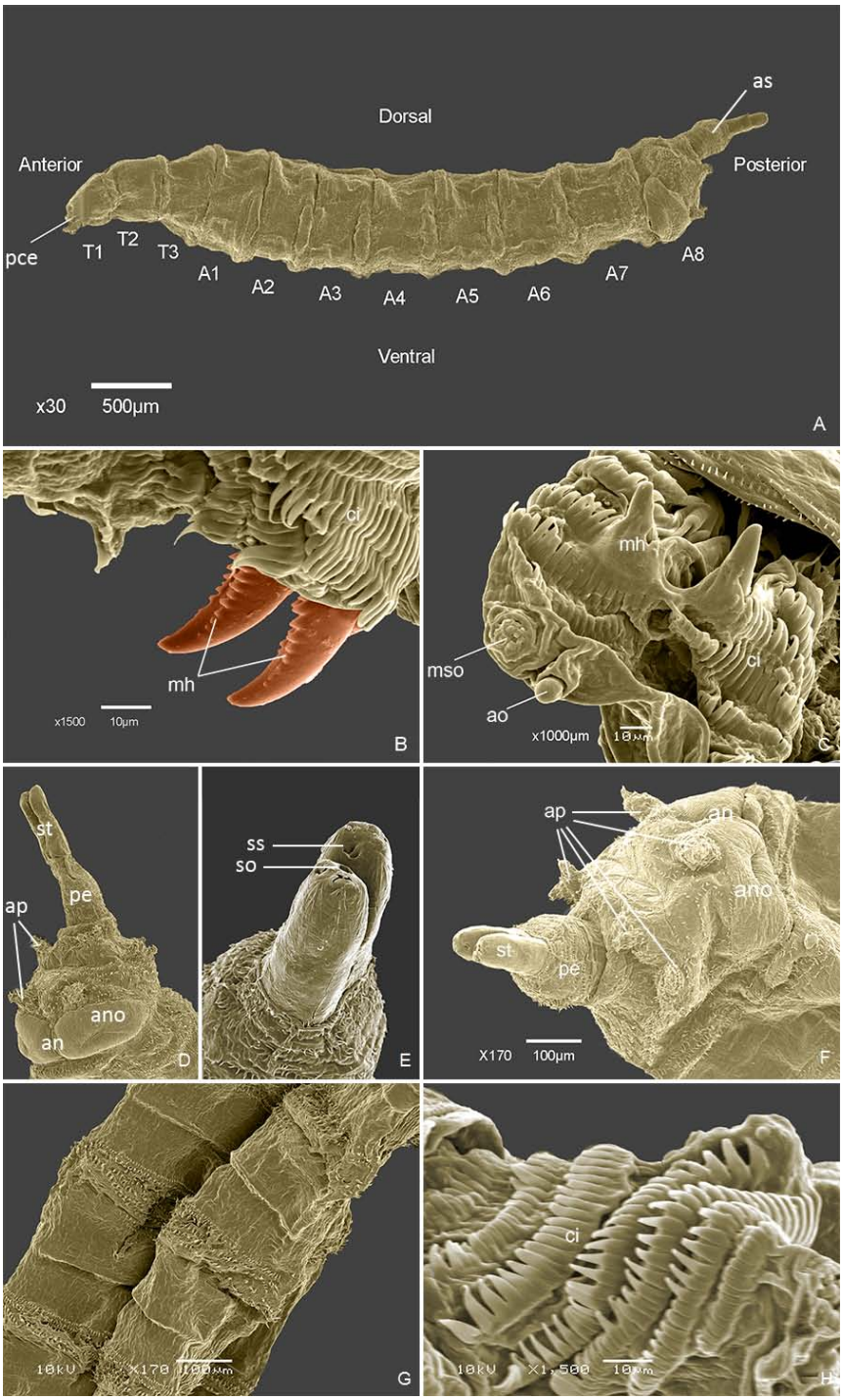

Figure 3. Scanning electron microscopy of the larvae of *Drosophila willistoni* species group.
A. Larvae in lateral view (*D. sucinea*), B. Oral hooks (*D. paulistorum* Amazonian), C. Anterior spiracles and oral hooks (*D. paulistorum* Amazonian), D. VIII. Abdominal segment, laterovental view (*D. insularis*), E. Posterior spiracles (*D. willistoni*), F. VIII. Abdominal segment, lateral view (*D. willistoni*), G. Cuticular denticles and hooks (*D. paulistorum* Amazonian) and H. Cirri (*D. sucinea*).
as, abdominal spiracle; pce, Pseudocephalon; mh, Mouth hooks; ci, Cirri; ao, Antennal organs; mso, Maxillar sensorial organs; st, Spiracular tube; pe, Peritrema; ap, Anal papilla; ano, Anal organs; an, Anus; so, Spiracular opening; ss, Spiracular scar.

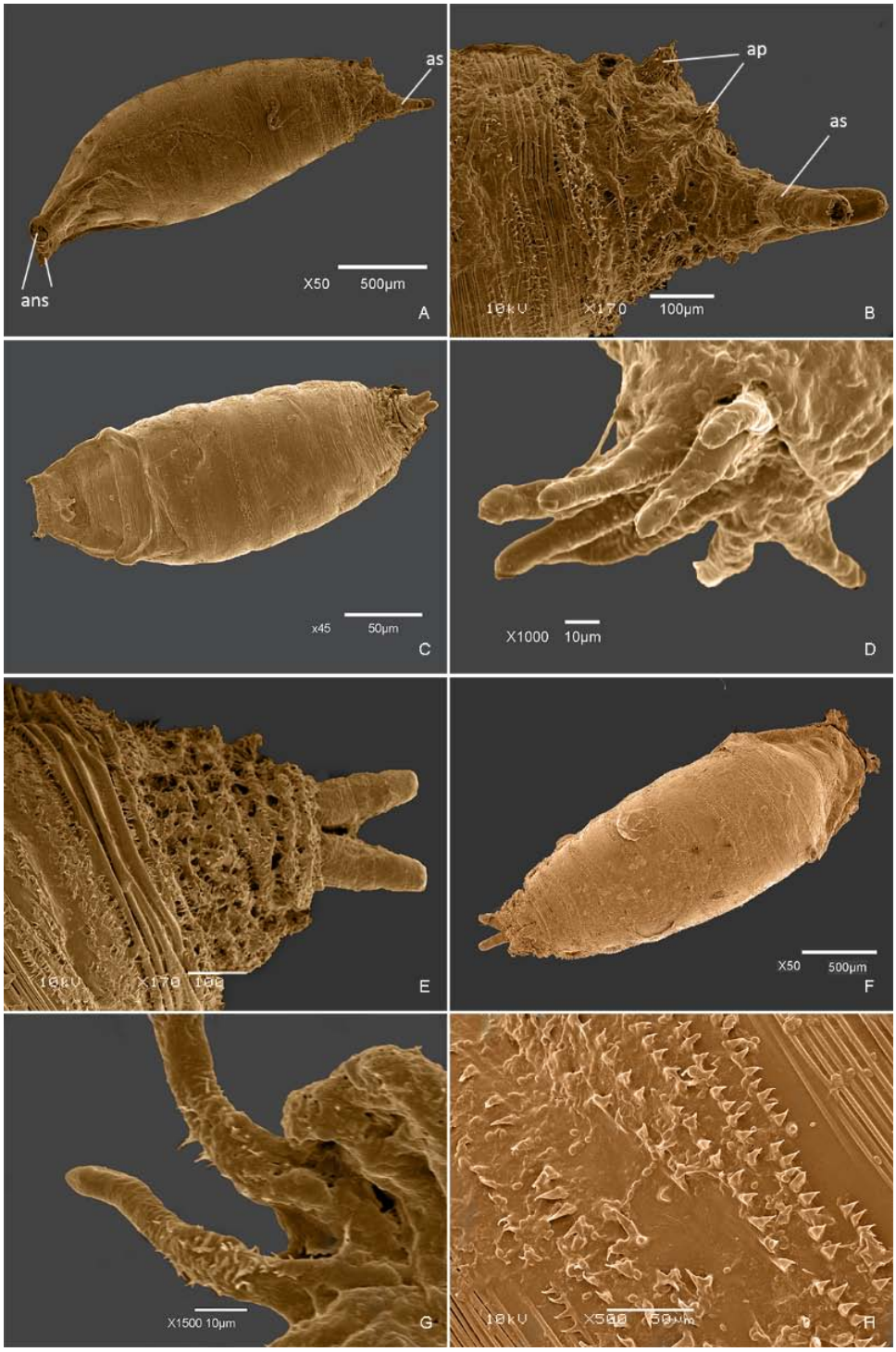

Figure 4. Pupae of *Drosophila willistoni* group.
A. Pupae in lateral view (*D. paulistorum* Andean-Brazilian), B. VIII Abdominal segment (*D. paulistorum* Andean-Brazilian), C. Pupae in ventral view (*D. paulistorum* Amazonian), D. Anterior spiracles (*D. capricorni*), E. Posterior spiracles, lateral view (*D. capricorni*), F. Pupae in dorsal (*D. paulistorum* Amazonian), G. Detail of anterior spiracles (*D. paulistorum* Interior) and H. Cuticular denticles (*D. sucinea*).
as, abdominal spiracle; ans, anterior spiracle; ap, anal papillae.

feature of pupae when comparing with different species. The anal segment of the pupae is very similar to that in the larvae, and it is possible to observe the anterior spiracles and anal papillae (Figures 4B, 4C, 4E and 4F).

As we observed for larvae, the external morphology of the pupae apparently does not vary among the *willistoni* group, even between the *bocainensis* and *willistoni* subgroups.

CONCLUSION

From the results obtained, we can infer that the analysis of the immature stages can be useful to differentiate *willistoni* subgroup from other species groups. However, those cannot be considered as diagnostic characteristics of the species when analyzed alone, since the level of differentiation is very low and seems to be restricted to the distal portion of the respiratory filaments of the eggs.

Evidences of intraspecific variation were not found, the same way as Zanini *et al.* [17] did not find intraspecific variation in the characters of male genitalia of *willistoni* subgroup. Intraspecific variation of the morphological characters of the immature stages were tested for two species and extrapolated for the remaining. Considering that even the interspecific differences were subtle (based on the shape of respiratory filaments), it is unlikely that with more lineages in the analysis, intraspecific differences would be found. The *D. willistoni* lineages included were from many different points from the distribution and therefore a gradient of phenotypes should be evident in case of intraspecific differences, which did not occur. Furthermore, besides the lack of marked differences within the *willistoni* subgroup and between this group and the species analyzed from *bocainensis* subgroup, we cannot state that this pattern is also true for the rest of the *bocainensis* subgroup or the *alagitans* subgroup, since those two were not analyzed in their entirety. In a further study, the immature stages of the remaining species of the *bocainensis* subgroup and species of *alagitans* subgroup will be analyzed.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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