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Eggshell as a characteristic to identify *Lutzomyia (Nyssomyia) intermedia* (Lutz & Neiva, 1912) and *Lutzomyia (Nyssomyia) neivai* (Pinto, 1926) (Diptera: Psychodidae: Phlebotominae), vectors of cutaneous leishmaniasis

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Wagner Alexandre Costa
Laboratório de Transmissores de Leishmanioses, Instituto Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brasil

Simone Miranda da Costa
Laboratório de Transmissores de Leishmanioses, Instituto Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brasil

Elizabeth Ferreira Rangel
Laboratório de Transmissores de Leishmanioses, Instituto Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brasil

Jacenir Reis dos Santos-Mallet
Laboratório de Transmissores de Leishmanioses, Instituto Oswaldo Cruz, Rio de Janeiro, Rio de Janeiro, Brasil

José Eduardo Serrão
Departamento de Biologia Geral, Universidade Federal de Viçosa, Viçosa, Minas Gerais, Brasil

ABSTRACT

Sand flies are the main vectors of leishmaniasis diseases that infect approximately 12 million people in 88 countries, with approximately 350 million people living in risk areas. Sand fly species are identified based on the morphology of the adults, and there are few studies of the immature insects, making the accurate identification of these vectors prior to adult emergence difficult. This study investigated the eggshell morphology of *Lutzomyia (Nyssomyia) intermedia* and *Lutzomyia (Nyssomyia) neivai*. The results obtained with scanning electron microscopy and quantitative analyses showed the occurrence of significant differences among numbers of tubercles in the eggshell of both species, besides the presence of some cross-conections between ridges, only in *L. (N.) intermedia*, thus enabling the morphological differentiation between eggs of both species.

Keywords: Psychodidae; Insect Vectors; Animal Structure; Scanning Electron Microscopy; Chorionic Sculpturing; Systematics.

INTRODUCTION

Sand flies are important to public health because they are the main vectors of leishmaniasis diseases, including American cutaneous leishmaniasis (ACL), which affects 25,500 individuals/year in Brazil[1]. The disease has different clinical and pathological features due to the diversity of the parasites and the immunity of the infected individuals[2,3,4]. To date, 45 species of sand flies are estimated to be associated with the transmission of cutaneous leishmaniasis in the New World[5,6].

Sand fly species are identified based on the morphology of the adult insects, and there are few studies of the immature stages. The immature stages of only approximately 70 species of the 510 described species of Neotropical sand flies have been well characterized[7,8,9,10,11,12].

Lutzomyia (Nyssomyia) intermedia and *Lutzomyia (Nyssomyia) neivai* are the main vectors of dermal leishmaniasis in Southern and Southeastern Brazil[4,5,13]. *L. (N.) intermedia* was described by Lutz & Neiva (1912) using adult specimens collected in Ouro Fino farm, in the City of Além Paraíba, Minas Gerais State. *L. (N.) intermedia* is considered as the first species described in

Correspondence / Correspondência / Correspondencia:
Jacenir Reis dos Santos-Mallet
Av. Brasil, 4365. Bairro: Manguinhos
CEP: 21040-360 Rio de Janeiro-Rio de Janeiro-Brasil
Tel.: +55 (21) 2562-1352
E-mail: jacenir@ioc.fiocruz.br

the Neotropics and was redescribed in 1996 by Marcondes[14]. *L. (N.) neivai* was described by Pinto (1926) using a male trapped inside a residence at the Instituto Butantan, in the City of São Paulo, São Paulo State. These species may occur together in the same place, especially in the Vale da Ribeira, São Paulo State. For several years, these two species were synonyms; however, morphological studies of different populations of *L. (N.) intermedia* in Brazil, showed that this species could be a species complex of *L. (N.) intermedia* sensu strictu together with *L. (N.) neivai*. The former species is found in regions of low altitude, and the second is adapted to cold and dry environments[14,15,16].

Although both species show some degree of genetic introgression, revisited *L. (N.) intermedia* and *L. (N.) neivai* were concluded to be valid species based on the following characteristics: the maximum width of the spermathecal rings, symmetry of annulation, head shape, length of the spermathecal duct and shape of the male genital filaments[15,17].

The advent of taxonomic tools based on such technological advances as scanning electron microscopy (SEM) since 1965 has been useful to clarify the taxonomic status of some species complexes[18,19,20,21,22]. Thus, the characterization of immature stages of insects using high-resolution tools may contribute to the accurate identification of sand fly species. This study investigated the eggshell ornamentation of the eggs of *L. (N.) intermedia* and *L. (N.) neivai*, showing that the eggs can be used to identify both of the species.

MATERIALS AND METHODS

INSECTS

The adult specimens were collected from areas with an occurrence of leishmaniasis in Southeast Brazil. *L. (N.) intermedia* was collected in Jacarepaguá, a Municipality of Rio de Janeiro, Rio de Janeiro State, and in the Municipality of Iporanga, São Paulo State. *L. (N.) neivai* was collected in the Planalto region of São Paulo State. Both species were collected using HP-type light traps[23].

Colonies of these two species were maintained in the Laboratório de Transmissores de Leishmanioses, Instituto Oswaldo Cruz and in the Escola de Saúde Pública, Universidade de São Paulo following described procedures[24,25].

SCANNING ELECTRON MICROSCOPY

A total of 80 eggs of each species was obtained from the colonies and transferred to a 2.5% glutaraldehyde solution in 0.1 M sodium cacodylate buffer (pH 7.2) for 1 h. The eggs were then post-fixed in 1% osmium tetroxide in the same buffer for 1 h at room temperature. After washing in the same buffer, the eggs were dehydrated in a graded ethanol series, dried in a critical point dryer with CO_2 (Balzers), covered with gold (20 nm) and analyzed using a LEO 1430 VP scanning electron microscope (SEM) at the Centro de Microscopia e Microanálise at the Universidade Federal de Viçosa, Minas Gerais State, Brazil, and using a Jeol 6390 LV in the Rudolf Barth Electron Microcopy Platform at Fundação Oswaldo Cruz, Rio de Janeiro State, Brazil.

MORPHOMETRY

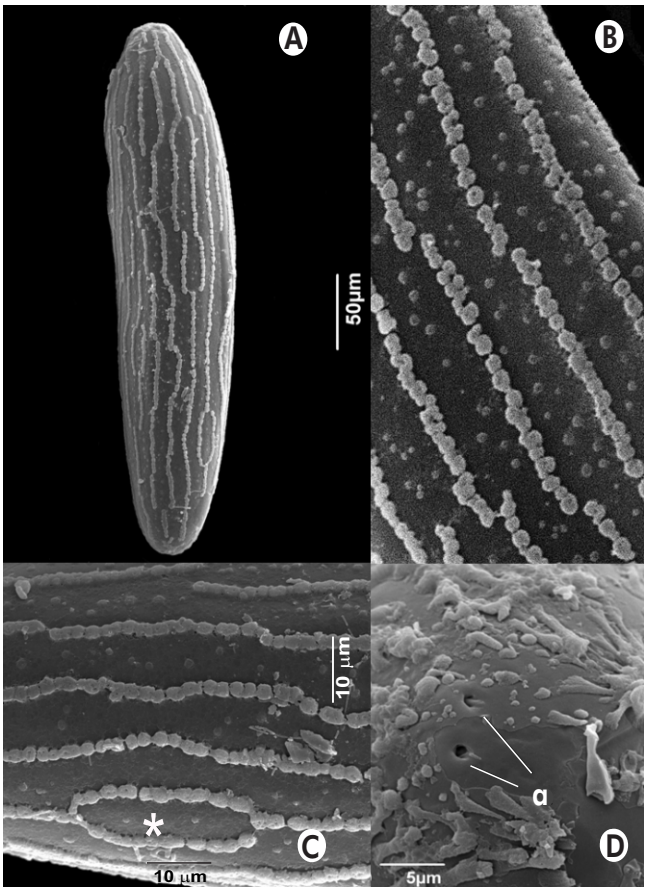
The SEM images were analyzed using the computer program SemAfore 3.0 (JEOL, Sollentuna, Sweden), and the number of eggshell tubercles was calculated considering a 1 μm^2 area/egg.

STATISTICAL ANALYSIS

The ANOVA test was applied at a significance level of 0.05 using SPSS 10.0 for Windows (SPSS Inc., 233 South Wacker Drive, 11th Floor, Chicago, IL).

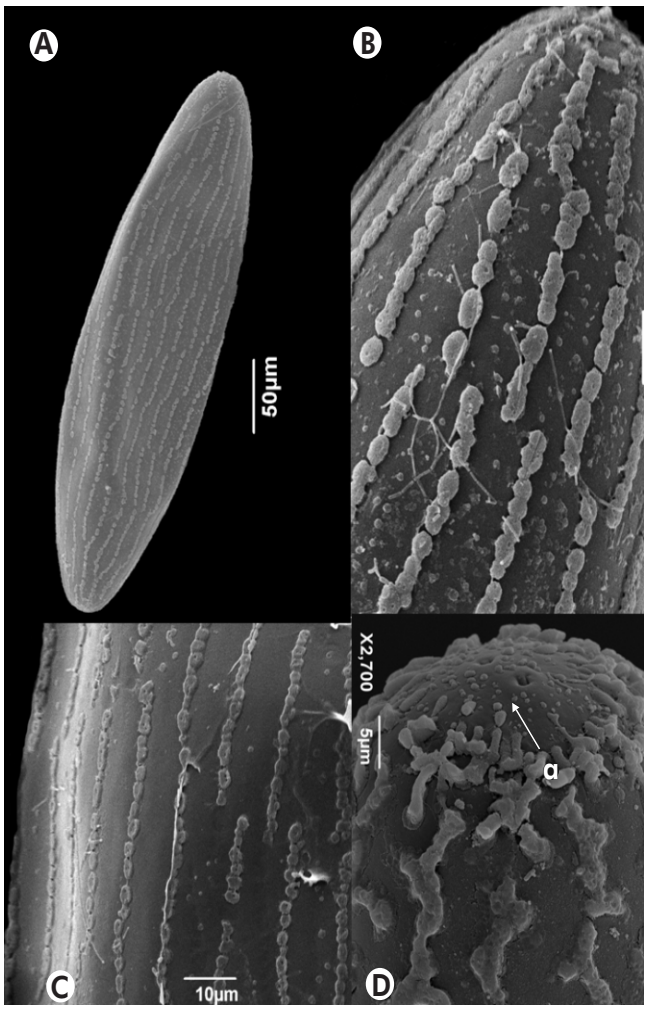
RESULTS

The eggs of *L. (N.) intermedia* and *L. (N.) neivai* were dark, elliptical, elongated and slightly flattened. The anterior and posterior regions of the eggs of both species were rounded, presenting two round aeropylar apertures at the end (Figures 1D, 2B and 2D). The *L. (N.) intermedia* eggs measured 361 μm in length and 75.4 μm in width in their central region; the *L. (N.) neivai* eggs measured 343 μm in length and 85.5 μm in width (Figures 1A and 2A).



A: General view of the egg showing an ornamentation characterized by the presence of low ridges in parallel arrays; B: Detail of the eggshell ornamentation showing parallel ridges, with small tubercles among them; C: Ridges connections of the eggs forming elliptical-looking areas (*); D: Aeropylar area of *L. (N.) intermedia;* a: aeropylar openings.

Figure 1 – Egg of the specie *L. (N.) intermedia* analyzed using a LEO 1430 VP and a Jeol 6390 LV SEM



A: General view of the egg showing slightly rounded structures arranged in rows, sometimes continuous or interrupted; B: Anterior region of the egg; C: Detail of the eggshell ornamentation showing ridges and small tubercles; D: Aeropylar area of *L. (N.) neivai;* a: aeropylar openings.

Figure 2 – Egg of the specie *L. (N.) neivai* analyzed using a LEO 1430 VP and a Jeol 6390 LV SEM

The exochorion showed an ornamentation characterized by the presence of low ridges, with parallel arrays along the long axis of the egg (Figures 1A and 2A), which were formed by slightly rounded structures arranged in rows that were continuous or interrupted (Figures 1B and 2C). The ridges had no cross-connections between them on the *L. (N.) neivai* eggs (Figures 2B and 2C), whereas some connections were observed on the *L. (N.) intermedia* eggs, forming elliptical-looking areas (Figure 1C). Small isolated tubercles were found between the spaces formed by the ridges (Figures 1B and 2B), varying in number between the two studied species: 8.55 ± 1.80 tubercles/1 μm^2 in *L. (N.) intermedia* and 5.95 ± 1.87 tubercles/1 μm^2 in *L. (N.) neivai*. When applying ANOVA, a difference was observed between the number of tubercles per 1 μm^2 (0,05 < p; p = 0,0).

DISCUSSION

Several studies investigating the surfaces of eggs have been conducted in Diptera with regard to taxonomic identification and oviposition sites[26,27,28,29,30,31]. SEM is a powerful tool that complements classical taxonomy, as many structures that are not visible using light microscopy may be important for the morphological characterization of insects[32,33,34,35].

The occurrence of structures on the *L. (N.) intermedia* and *L. (N.) neivai* exochorion characterized by the occurrence of longitudinal ridges without connections between each other is a character commonly found in sand flies. A study of five sand fly species reported parallel ridges forming polygons at irregular intervals in *L. caballeroi* or connected ridges and a reticular pattern in *L. peruensis*. Some of these ridges are subdivided to form elliptical or hexagonal areas[20,22], similar to our observations on some regions of the chorion of *L. (N.) intermedia*. In contrast, the authors of the above study observed an undefined pattern in *L. tejedai*. Our results show that it is possible to identify *L. (N.) intermedia* and *L. (N.) neivai* based on the eggshell morphology, similar to the sand flies Phlebotomus and Sergentomyia[36], corroborating that the ornamentation of the exochorion is a good characteristic for species identification in Diptera[27,28].

Considering the origin and function of the exochorion, some authors have suggested the existence of a relationship between the species and their larval development in micro-ecosystems and that the eggshell ornamentations are adaptive structures that facilitate oviposition in different environments[18,20]. The chorion is secreted by the ovarian follicular epithelial cells during choriogenesis, and its viscosity allows the egg to adhere to the substrate. The chorion is a rigid structure forming the eggshell and is composed of two layers: an inner layer, the endochorion in contact with the embryo, and an outer layer, the exochorion in contact with the environment[36]. When desiccated, the chorion presents distinct layers, generally forming elaborate structures on the surface of the egg, with the patterns being characteristic for each species. This layer serves to cover and protect the embryo, preventing it from desiccation and regulating gaseous exchange[37,21,38]. Since *L. (N.) intermedia* is found in warm and humid environments, whereas *L. (N.) neivai* is found in cold and dry environments possible association between differences on egg surface of both species to enviroments should be evaluated. In addition to studies on the ecological adaptations, the eggshell structures of these species may contribute to future investigations of the phylogenetic relationships of the genus *Lutzomyia*, as suggested for other representative sand flies[37,19,20,21,38].

CONCLUSION

The study of the immature insect forms by SEM produces a deeper knowledge of the different species of sandflies, with the purpose of giving a precise identification based on the immature stages.

Our findings using scanning electron microscopy and the quantitative analysis of the eggshell tubercles showed a variation of this structure that enables a morphological differentiation between *L. (N.) intermedia* and *L. (N.) neivai*.

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Cascas de ovos como um fator de identificação de *Lutzomyia (Nyssomyia) intermedia* (Lutz & Neiva, 1912) e *Lutzomyia (Nyssomyia) neivai* (Pinto, 1926) (Diptera: Psychodidae: Phlebotominae), vetores da leishmaniose tegumentar

RESUMO

Os flebotomíneos são os principais vetores das leishmanioses. Essas doenças atingem aproximadamente 12 milhões de pessoas em 88 países, e cerca de 350 milhões de pessoas vivem em áreas de risco. As espécies de flebotomíneos são identificadas com base na morfologia de seus espécimes adultos. A existência de poucas pesquisas sobre espécimes imaturos torna difícil uma identificação exata desses vetores antes de atingirem a idade adulta. Este estudo investigou a morfologia das cascas de ovos das espécies *Lutzomyia (Nyssomyia) intermedia* e *Lutzomyia (Nyssomyia) neivai*. Os resultados obtidos por meio de microscopia eletrônica e análises quantitativas demonstraram a ocorrência de diferenças significativas entre os números de tubérculos nas cascas de ovos de ambas as espécies, além da presença de algumas conexões cruzadas entre cristas apenas nos espécimes de *L. (N.) intermedia*, possibilitando assim a diferenciação morfológica de seus ovos.

Palavras-chave: Psychodidae; Insetos Vetores; Estrutura Animal; Microscopia Eletrônica de Varredura; Escultura Coriônica; Sistemática.

Cáscaras de huevos como un factor de identificación de *Lutzomyia (Nyssomyia) intermedia* (Lutz & Neiva, 1912) y *Lutzomyia (Nyssomyia) neivai* (Pinto, 1926) (Diptera: Psychodidae: Phlebotominae), vectores de la leishmaniasis tegumentaria

RESUMEN

Los flebótomos son los principales vectores de las leishmaniasis. Esas enfermedades alcanzan a aproximadamente 12 millones de personas en 88 países, y cerca de 350 millones de personas viven en áreas de riesgo. Las especies de flebótomos se identifican con base en la morfología de sus especímenes adultos. La existencia de pocas investigaciones sobre especímenes inmaduros torna difícil una identificación exacta de esos vectores antes de alcanzar la edad adulta. Este estudio investigó la morfología de las cáscaras de huevos de las especies *Lutzomyia (Nyssomyia) intermedia* y *Lutzomyia (Nyssomyia) neivai*. Los resultados obtenidos a través de microscopia electrónica y análisis cuantitativos demostraron la ocurrencia de diferencias significativas entre los números de tubérculos en las cáscaras de huevos de ambas especies, además de la presencia de algunas conexiones cruzadas entre cristas apenas en los especímenes de *L. (N.) intermedia*, posibilitando así la diferenciación morfológica de sus huevos.

Palabras clave: Psychodidae; Insectos Vectores; Estructura Animal; Microscopía Electrónica de Barridoa; Escultura Coriónica; Sistemático.

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