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PROGRAMA DE PÓS-GRADUAÇÃO STRICTO SENSU EM BIOLOGIA DA INTERAÇÃO PATÓGENO HOSPEDEIRO

ERIC FABRÍCIO MARIALVA DOS SANTOS

BIONOMIA DE *Migonemyia migonei* (DIPTERA, PSYCHODIDAE, PHLEBOTOMINAE) EM CONDIÇÕES EXPERIMENTAIS

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ORIENTADOR: Prof. Dr. Felipe Arley Costa Pessoa

CO-ORIENTADORA: Dra. Nágila Francinete Costa Secundino

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RESUMO

A espécie *Migonemyia migonei* é vetora de *Leishmania braziliensis* no Brasil. Estudos recentes demostraram que essa espécie pode estar participando do ciclo de transmissão de *Leishmania infantum chagasi* no Brasil e Argentina. O presente estudo teve como objetivo estudar a biologia de *Mi. migonei*, verificando morfologia dos imaturos, aspectos de colonização em massa e desenvolver modelo de transmissão de *Le. in. chagasi* através da picada da mesma, de indivíduos colonizados. Para alcançar esses objetivos foram feitos vários experimentos, os estágios imaturos (ovo, larva e pupa) foram analisados por MEV e microscopia de luz; colonização em massa foi verificado ciclo de vida, longevidade, fecundidade, fertilidade e preferência de oviposição em substratos; para transmissão, fêmeas de *Mi. migonei* foram infectadas com *Le. in. chagasi* e *Le. braziliensis* (controle) de isolados em meio de cultura; , posteriormente fêmeas infectadas foram colocadas para picar camundongos. Como resultado da descrição, os ovos de *Mi. migonei* apresentam um exocório com estruturas poligonais, a quetotaxia das larvas e pupas apresentaram diversas cerdas diferenciadas de outras espécies. O ciclo de vida de ovo até emergência do adulto foi de 62,1 dias em média. A maior fertilidade e fecundidade foi com o sangue de hamster (36,68, $p < 0.05$), seguido de humano (23,28), camundongo (19,63) e pinto (12,20). Fêmeas de *Mi. migonei* alimentadas com maçã sobreviveram maior tempo (10 dias) seguido de água açucarada a 10% (7 dias) e água (5 dias). Não foi observado diferença na taxa de oviposição dos diferentes substratos. Quanto a transmissão *Mi. migonei* foi capaz de transmitir *Le. in. chagasi*, a quantidade de parasita variou de 10 a 1000 em uma única orelha. Estes resultados contribuem para biologia de *Mi. migonei* e desvendar seu papel como vetora na transmissão de *Le. in. chagasi*.

Palavras Chave: taxonomia, colonização, espécie vetora.

ABSTRACT

The species *Migonemyia migonei* is a vector of *Leishmania braziliensis* in Brazil. Recent studies have shown that this species may be participating in the transmission cycle of *Leishmania infantum chagasi* in Brazil and Argentina. The present study had as objective to study the biology of *Mi. migonei*, verifying the morphology of the immature, aspects of mass colonization and to develop a model of transmission of *Le. in. chagasi* through the sting of it. In order to reach these objectives, several experiments were carried out, the immature stages (egg, larva, and pupa) were analysed by MEV and light microscopy; colonization in mass was verified life cycle, longevity, fecundity, fertility and preference of oviposition in substrates; for transmission *Mi. migonei* was infected with *L. in. chagasi* and *Le. braziliensis* (control), was later placed to prick mice. The eggs of *Mi. migonei* present an exochorion with polygonal structures, which chaetotaxy of larvae and pupae presented differentiated bristles of other species. The egg life cycle up to adult emergence was 62.1 days on average. The highest fertility and fecundity was with hamster blood (36.68, $p < 0.05$), followed by the human (23.28), mouse (19.63) and chick (12.20). *Mi. migonei* females fed with apple survived longer (10 days) followed by 10% water (7 days) and water (5 days). No difference was observed in the oviposition rate of the different substrates. As for transmission *Mi. migonei* was able to transmit *Le. in. chagasi*, the amount of parasite varied from 10 to 1000. These results may contribute to the biology of *Mi. migonei* and its role as a vector in the transmission of *Le. in. chagasi*.

Keywords: taxonomy, colonization, vector species.

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1 Introdução

1.1 Leishmanioses

As leishmanioses encontram-se entre as seis endemias mais prevalentes transmitidas por insetos no mundo; aproximadamente um bilhão de pessoas encontram-se sob risco de contrair leishmaniose, desses, 616 milhões de contrair leishmaniose visceral (LV) e 431 milhões para leishmaniose tegumentar (LT) (WHO 2016).

A manifestação clínica da LT, se apresenta, em geral, em forma de lesões que acometem a pele e mucosa. As lesões de pele podem ser únicas, múltiplas, disseminadas ou difusas. As lesões nas mucosas são mais frequentes no nariz, boca e garganta (Neves 2004, Brasil 2017). No mundo, em 2017, foram notificados 142.635 casos de LT. No Brasil foram 17.809 casos de leishmaniose tegumentar (WHO, 2017). De acordo com dados do Portal Saúde do Governo Federal (Brasil 2019), a região Norte apresentou 7.832 casos registrados, sendo 1.865 casos para o estado do Amazonas.

No Brasil há sete espécies de *Leishmania* Ross, inseridas em dois subgêneros- *Leishmania (leishmania)* e *Leishmania (Viannia)* que causam leishmaniose tegumentar em humanos, a *Leishmania guyanensis* Floch, *Le. amazonensis* Lainson e Shaw, *Le. braziliensis* (Vianna), *Le. lainsoni* Silveira, Shaw, Braga e Ishikawa, *Le. shawi* Lainson, Braga e de Souza, *Le. naiffi* Lainson e Shaw e *Le. lindenbergi* Silveira, Ishikawa e De Souza. Essas espécies de leishmânias são transmitidas por diferentes espécies de vetores. *Le. guyanensis* é transmitida por *Nyssomyia umbratilis* Ward e Fraiha, *Ny. anduzei* Rozeboom e *Ny. whitmani* Antunes E Coutinho; *Le. amazonensis* por *Ny. flaviscutellata* (Mangabeira) e *Lutzomyia longipalpis* (Lutz e Neiva); *Le. braziliensis* por *Ny. whitmani* (Antunes e Coutinho), *Ny. intermedia* (Lutz e Neiva), *Psychodopygus wellcomei* Fraiha, Shaw e Lainson, *Ps. complexa* (Mangabeira), *Ny. neivai* (Pinto), *Evandromyia edwardsi* (Mangabeira) e *Migonemyia migonei*; *Le. lainsoni* por *Trichophoromyia ubiquitalis* (Mangabeira); *Le. shawi* por *Ny. whitmani*; *Le. naiffi* por *Ps. amazonensis* (Root), *Ps. paraensis* (Costa Lima), *Ps. ayrozai* (Barretto & Coutinho), *Ps. squamiventris* (Lutz & Neiva); o vetor de *Le. lindenbergi* ainda não é conhecido (WHO, 2012).

A LV manifesta sob a forma visceral, que os parasitos causam febre de longa duração, aumento do fígado e baço, perda de peso, fraqueza, redução da força muscular e anemia (Neves 2004, Brasil 2017). Esta doença é um problema de saúde pública

mundial, com 300 mil novos casos anuais e 20 mil mortes por ano (WHO, 2015). No mundo, em 2017, foram notificados 22.145 casos de LV. No Brasil foram 4.297 casos de LV (WHO, 2017). De acordo com dados do Portal Saúde do Governo Federal, a região Nordeste a mais afetada apresentou 1.824 registros.

No Brasil, *Leishmania infantum chagasi* é a espécie responsável em causar LV, essa espécie de leishmânia é transmitida por *Lutzomyia longipalpis*. Alguns estudos especularam que essa espécie de leishmânia pode ser transmitida por outros vetores, como *Migonemyia migonei* que é constantemente encontrada com DNA de *Le. in. chagasi*, em área endêmica no Brasil (Carvalho et al. 2007, Carvalho et al 2010, Silva et al. 2014, Rodrigues et al. 2016) e na Argentina (Salomón et al. 2010, Moya et al. 2015). Outra espécie considerada vetora é a *Lutzomyia cruzi* (Mangabeira), onde Santos et al. (1998) em estudo em Mato Grosso do Sul, verificaram por dissecação que indivíduos de *Lu. cruzi* apresentaram em seu intestino promastigotas de *Le. in. chagasi*.

1.2 Flebotomíneos

Os flebotomíneos são da ordem Diptera, família Psychodidae e subfamília Phlebotominae, apresentam o desenvolvimento holometábolo, passando pelas fases de ovo, larva (quatro estádios), pupa e adulto. Em condições de laboratório seu ciclo de vida pode variar de 30 a 100 dias, dependendo da espécie e das condições de temperatura e umidade (Foratini 1973). Ferro et al (1998) verificaram que o ciclo de vida de uma espécie neotropical de flebótomo, a *Psathyromyia shannoni*, em laboratório durou 54,6 dias em média. Em estudos com outras espécies neotropicais de flebotomíneos em condições de laboratório, foi verificado que *Lutzomyia longipalpis*, *Tricholateralis cruciata* e *Nyssomyia umbratilis* respectivamente tiveram um ciclo de vida de 35,9; 52,7 e 75,14 dias em média (Brazil et al. 1997, Castillo et al. 2015, Justiniano et al. 2004). Recentemente Lawier et al. (2017) em uma revisão sobre estudo com criação em laboratório de flebotomíneos, verificaram que as espécies paleárticas *Phlebotomus perniciosus, Ph. longicuspis* e *Ph. perfiliewi* seus ciclos de vida variaram entre 46 e 48 dias em média.

A fase de ovo de flebotomíneos em condições de laboratório dependendo da espécie pode dura de sete a 10 dias; o período larval pode durar de 21 dias até empuparem e de sete a 14 dias para emersão dos adultos (Queiroz 1995). Brazil & Brazil (2018) estudaram espécies neotropicais em condições de laboratório, verificaram que em média os ovos de flebotomíneos na temperatura de 25º C- 27ºC iniciaram sua

eclosão entre cinco a oito dias, indo até ás vezes, mais de 15 dias para tal, o período larval pode vir a durar de 20 a 35 dias, já o período pupal de quatro a 10 dias. Já Lawier et al. (2017) estudaram espécies paleárticas em condições de laboratório e verificaram que os ovos eclodiram entre seis e 11 dias, mas em condições desfavoráveis ficaram em diapausa em até 30 dias ou mais, a fase larval duraram de 21 a 25 dias para empuparem e 10 dias para emersão dos adultos.

Estudos de biologia de flebotomíneos são importantes pois ainda há grandes lacunas no conhecimento da biologia da maioria das espécies principalmente nas de importância médica. O conhecimento sobre tempo de vida e incrementos de técnicas para aumentar longevidade são importantes para estudos de interação com *Leishmania* e outros patógenos (Killick-Kendrick et al. 1977).

Os adultos de flebotomíneos, necessitam de carboidratos como fonte de energia para exercerem as suas atividades de voo, acasalamento, postura e outras funções metabólicas; na natureza eles obtêm de néctares, seivas, e secreções açucaradas de afídeos (Homoptera) (Cameron et al. 1995, Brazil & Brazil 2018, Lawier et al. 2017). As fêmeas de flebotomíneos realizam hematófagia para maturação de seus ovos. Por serem hematófagas, as fêmeas de flebotomíneos transmitem diversos patógenos, incluindo protozoários da ordem Kinetoplastida, família Trypanosomatidae do gênero *Leishmania* que causam as leishmanioses.

Para manutenção de flebótomos adultos, em condições de laboratório, são oferecidos diferentes fontes de carboidratos dependendo da espécie de flebotomíneo colonizada. Chaniotis (1967) oferecia como fonte de carboidrato uvas passas e água açucarada a 10% para a espécie *Helcocytomyia stwarti* (Mangabeira Fo & Galindo). Mann & Kaufman (2010) ofereceram para a espécie *Ps. shannoni*, maçã ou água açucarada a 20% como fonte de carboidrato. Em trabalho recente com colonização de *Ny. neivai* Gourlat et al. (2017) ofereceram como fonte de carboidrato água açucarada a 30%. Estudos de fonte de carboidratos para flebotomíneos são importantes, pois auxiliam na manutenção e vitalidade de flebotomíneos em condições de laboratório.

Colônias de flebotomíneos mantidas em laboratório são de valor inestimável para poder conhecer sua biologia, fisiologia, comportamento, competência vetorial, interação parasito-hospedeiro, suscetibilidade, resistência a inseticidas e para elucidação de aspectos taxonômicos e outros (Queiroz 1995, Lawier et al. 2017). Para a realização desses tipos de estudos é preciso manter colônias estáveis, de forma que permitam obter

um grande número de indivíduos, o suficiente para a execução de vários tipos de experimentos (Killick-Kendrick 1977, Buescher et al. 1984, El Naiem e Ward 1992).

1.3 Migonemyia migonei

A espécie *Mi. migonei* foi descrita por França (1920), apenas os adultos, e as formas imaturas foram descritas parcialmente por Barreto (1941), através de microscopia de luz. Ela é incriminada como espécie vetora de *Leishmania braziliensis* (Araujo Filho 1979, Rangel & Lainson 2009). Essa espécie tem uma ampla distribuição pela América do Sul; no Brasil a *Mi. migonei* está presente nos estados do Acre, Amapá, Amazonas, Pará, Rondônia, Tocantins, Alagoas, Maranhão, Ceará, Paraíba, Pernambuco, Bahia, Rio Grande do Norte, Goiás, Mato Grosso, Mato Grosso do Sul, Espirito Santo, Minas Gerais, Rio de Janeiro, São Paulo, Paraná, Rio Grande do Sul e Santa Catarina (Aguiar & Vieira 2018) .Também está presente em outros países como Colômbia, Venezuela, Argentina, Paraguai, Peru e Trinidad e Tobago (Rangel & Lainson 2003; Rangel e Shaw 2018).

Alguns autores especularam que que essa espécie também está associada com transmissão de *Le. in. chagasi,* no Brasil e na Argentina. Carvalho et al. (2007), verificaram em São Vicente Férrer, Pernambuco que é uma área endêmica de LV, que *Mi. migonei* foi a segunda espécie mais abundante. Silva et al. (2014) sugeriram que *Mi. migonei* juntamente com *Lu. longipalpis* eram os vetores de *Le. in. chagasi* em Fortaleza no Ceará. Salomón et al. (2010) especularam que a *Mi. migonei* seria vetor putativo de *Le. in. chagasi* em um surto de LV em La Banda na Argentina. Na Argentina, Moya et al. (2015) detectaram por PCR , DNA de *Le. in. chagasi.* em espécimes de *Mi. migonei* coletados em campo. Em Fortaleza, Rodrigues et al. (2016) detectaram por qPCR, DNA de *Le. in. chagasi* em *Mi. migonei*, indicando ser um potencial vetor dessa leishmânia.

Guimarães et al. (2016) em experimentos de infecção de *Mi. migonei* com *Le. in. chagasi* em laboratório, constataram que essa espécie de flebotomíneo é permissiva à infecção dessa espécie de leishmânia. Nieves & Pimenta (2000) em estudo com infecção experimental de *Mi. migonei*, verificaram que essa espécie suportava a infecção de *Leishmania amazonensis*. Em estudo recente com infecção experimental, Nogueira et al. (2016) constataram que *Mi. migonei* foi capaz de sustentar a infecção de duas linhagens de *Le. amazonensis,* que apresentavam polimorfismos em seus

lipofosfoglicanos (LPG). Demostrando que essa espécie pode ser suscetível a diferentes espécies e linhagens de *Leishmania.*

1.4 Interação leishmânia-flebotomíneo

As espécies de *Leishmania* humanopatogênicas estão divididas em três subgêneros: *Leishmania, Viannia* (Lainson & Shaw, 1987) e *Mundinia* (Shaw, Camargo e Texeira). As espécies do subgênero *Viannia*, da qual faz parte *Le. braziliensis* desenvolvem-se inicialmente na região posterior do intestino do flebotomíneo sendo considerado peripilárico, já espécies do subgênero *Leishmania*, estão restrita a região suprapilárica do intestino do vetor, onde *Le. in. chagasi* faz parte (Lainson & Shaw, 1987), quanto espécies do subgênero *Mundinia* pouco se sabe quanto ao seu desenvolvimento em flebotomíneos (Rangel e Shaw, 2018).

A metaciclogênese da *Leishmania* no flebotomíneo se inicia quando a fêmea ao picar o hospedeiro vertebrado, ingere as formas amastigotas juntamente com o sangue. Imediatamente, o alimento sanguíneo começa a ser envolvido por uma estrutura secretada pelo epitélio intestinal, a matriz peritrófica (MP). A MP, composta de quitina e glicoproteína, envolve o alimento sanguíneo dentro do estômago do flebotomíneo após a ingestão de sangue (Walters et al. 1993, Pimenta et al. 1997, Secundino, 2005).

Em *Leishmania major, Le. braziliensis* e *Le. amazonensis*, dentro do vetor, a transformação das formas amastigotas em formas promastigotas procíclicas ocorre dentro de 12-24 horas após o repasto. Esses flagelados iniciam então um processo de divisão no intestino médio antes de se transformarem em formas alongadas denominadas nectomônadas, o que ocorre após 2-5 dias. Quando o sangue digerido é excretado após 3-5 dias da alimentação infectiva, as formas nectomônadas são encontradas no intestino médio. Esta migração é acompanhada por uma transformação em formas haptomônadas e promastigotas com núcleo e cinetoplasto justapostos. Estas formas estabelecem uma infecção maciça na válvula do estomodeu, com vários parasitos aderidos ao epitélio através de hemidesmossomas. Durante o estágio final da infecção, ocorre movimento das promastigotas em direção ao esôfago e faringe. A invasão do intestino anterior é acompanhada pelo aparecimento de formas pequenas, delgadas e altamente ativas denominadas promastigotas metacíclicas (Sacks 1989). Estas últimas podem ser injetadas no repasto sanguíneos seguintes, sendo capazes de iniciar infecção em um hospedeiro mamífero (Nieves & Pimenta 2000, Sacks & Kamhawi 2001, Kamhawi 2006).

A *Leishmania* no intestino do flebótomo estabelece uma infecção maciça na válvula estomodeu, essa infecção danifica essa válvula fazendo o flebotomíneo nos seguintes repasto regurgitar e fazer a maior propagação da *Leishmania*. Serafim et al. (2018) verificaram que *Ph. papatasi* e *Lu. longipalpis* infectados por *Le. major* e *Le. in. chagasi* respectivamente, a partir de um segundo ou mais repasto pós infecção, a quantidade de *Leishmania* infectivas aumentavam em seus intestinos.

Estudos de infecção experimental são importante, pois ajudam a compreender a biologia da interação entre parasitas e vetores e de incriminação vetorial, que não são respondidas em estudos de campo.

2 Justificativa

A *Mi. migonei* é uma espécie de ampla distribuição geográfica, dentro de área de transmissão de leishmaniose tegumentar e visceral, em áreas urbanas, com registros de infecção natural dessa espécie para espécies de *Leishmania* que causam a LT e LV.

A *Mi. migonei* é uma espécie permissiva à infecção por *Le. in. chagasi*. Estudos de campo indicaram que está ocorrendo uma mudança na dinâmica vetorial da LV, podendo ter outras espécies de flebótomos além de *Lu. longipalpis* como vetora. São necessários estudos de transmissão experimental de *Le. in. chagasi* em *Mi. migonei*, para se constatar a especulação feita por estudos de campo de incriminação vetorial em áreas de LV que não ocorrem a presença de *Lu. longipalpis*.

Para experimentos de infecção e transmissão de *Leishmania*, são necessários grande quantidade de flebotomíneos (Freitas et al. 2012). Estabelecimento e manutenção de colônias de flebotomíneos são difíceis e trabalhosas (Killick-Kendrick 1977, Lawier et al. 2017). No mundo existem apenas duas colônias de *Mi. migonei*, uma em Praga, República Tcheca, e outra em Manaus ambas originadas de mesma localização geográfica, na Serra de Baturité (Lawier et al. 2017), em uma área de transmissão mista de LT e LV, no Ceará (Queiroz et al. 1994).

Os estágios imaturos de *Mi. migonei* foram descritos por Barreto 1941, utilizando apenas microscopia de luz, outros autores estudaram essas fases imaturas já utilizando MEV, Fausto et al., 2001 e Almeida et al., 2004 verificaram o exocório dos ovos, Fausto et al. (1998) e Pessoa et al (2000) descreveram os espiráculos larvais e Pessoa et al. (2001) descreveram as antenas larvais. Depois de Barreto 1941, não foi feito nenhum trabalho que reúna a descrição das formas imaturas (ovo, larvas e pupa) dessa espécie vetora.

Esta dissertação propõe *i*- descrever as formas imaturas de *Mi. Migonei, ii*- estudar a biologia em laboratório e aprimorar as técnicas de criação de *Mi. migonei* de produção em massa e *iii*- compreender a transmissão de *Le. in. chagasi* por *Mi. migonei* em modelo murino.

3 Objetivos

Geral: Avaliar a bionomia de *Migonemyia migonei* (Diptera, Psychodidae, Phlebotominae) em condições experimentais.

Específicos:

1. Descrever as formas de ovo, larva e pupa de *Mi. migonei;*
2. Descrever o ciclo de vida de *Mi. migonei* criado em laboratório;
3. Avaliar o efeito de diferentes fontes sanguíneas sobre a fertilidade e fecundidade de *Mi. migonei;*
4. Avaliar o efeito dos diferentes substratos de estímulo para oviposição de *Mi. migonei*;
5. Avaliar o efeito de diferentes fontes de carboidratos na longevidade de *Mi. migonei*;
6. Avaliar o modelo de transmissão de *Leishmania infantum chagasi* usando *Mi. migonei* como vetor.

Artigo 1

Description of immature stages of *Migonemyia migonei* (França) (Diptera: Psychodidae, Phlebotominae) an important vector of Leishmanioses in South America under light and scanning electron microscopy

Description of immature stages of *Migonemyia migonei* (França) (Diptera: Psychodidae, Phlebotominae) an important vector of Leishmaniosis in South America under light and scanning electron microscopy

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Abstract

The sand fly systematics has been mainly based on adult morphological characters and few larval or pupal characters have been used. It is important to study these flies due to the vector importance of leishmaniasis, bartonellosis and arboviruses transmission due to their hematophagous habits. In this work, we re-described the immature stages of *Migonemyia migonei*, the vector of *Leishmania (Viannia) braziliensis*, the aetiological agent of Cutaneous Leishmaniasis in Brazil and other countries of South America and a putative vector of *Leishmania infantum chagasi*. The Scanning electron microscopy (SEM) and light microscopy were used to redescribe the specie and describe new characters. The eggs present exochorion with polygonal crosslinking, the first instar larva antenna different from the other instars, the 4th instar larva has a bristle "B" near the posterior spiracle and the pupa with six setae in the VIII abdominal segment, one in the spiracle and one adjacent to it, the setae 2 and 3 in the abdominal segments are inserted in large tubers. These results contribute to the taxonomy of *Mi. migonei* and may contribute to future studies of the phylogeny of the species.

Keywords: larva, pupae, chaetotaxy, morphology, ontogeny

Introduction

The knowledge about several aspects of the immature stages of phlebotomine sand flies (Diptera: Psychodidae) is still a challenge, due to the difficulties of finding a natural breeding site or lab colonization. So, of about more than 536 species described in the Neotropics (Godoy *et al*., 2017; Shimabukuro *et al*., 2017; Galati 2018; Posada-López *et al*., 2018; Szelag *et al*., 2018; Oliveira *et al*., 2018), larval stages of 93 species

of the New World sand flies have been described, or partially described (Table 1). The knowledge about larval structures is important due to the importance to give some highlights about taxonomy, phylogeny, and evolutions of this subfamily. In the last few decades, new proposals in phylogeny sand flies, especially based on adult morphology has been highlighted and several authors have adopted the Galati proposal (Galati 1994), that changed the taxonomy, phylogeny and nomenclature basis of phlebotomine systematic.

The use of scanning electron microscopy (SEM) improved the descriptions, providing details of larval chaetotaxy (Leite & Williams, 1996, 1997); ontogeny (Secundino & Pimenta, 2000; Bahia *et al*., 2007; Oca-Aguilar, 2014); spiracles (Fausto *et al.*, 1999; Pessoa *et al*.,2000); antennal, mouthparts sensillae and caudal setae descriptions (Bahia *et al*., 2007, Pessoa *et al*, 2001, 2008). Just a few articles appear has been published about pupal morphology of new world phlebotomine (Leite *et al*., 1991; Ocar-Aguilar *et al*., 2014; Ocar-Aguilar *et al*., 2016; Ocar-Aguilar *et al*., 2017; Alencar *et al*., 2018). Apart from several papers mentioned above, the number of descriptions of immature forms of sand flies is still scarce.

Table 1. Immature species of the Neotropical region described and their authors.

Species	Authors
Bichromomyia flaviscutellata	Ward, 1976
Bichromomyia olmeca bicolor	Hanson, 1968
Bruchomyia. argentina	Satchell 1953
Brumptomyia avellari	Mangabeira, 1942
Brumptomyia brumpti	Carneiro e Sherlock, 1964
Brumptomyia galindoi	Hanson, 1968
Brumptomyia guimaraesi	Barreto, 1941
Brumptomyia travassosi	Mangabeira, 1942
Brumptomyia. hamata	Hanson, 1968
Dampfomyia beltrani	Oca-Aguilar et al 2014
Dampfomyia insolita	Hanson, 1968
Dampfomyia isovespertilionis	Hanson, 1968
Dampfomyia vespertilionis	Hanson, 1968
Deanemyia maruaga	Alencar 2012
Deanemyia samueli	Alencar 2012
Deanemyia. derelicta	Alencar 2012
Evandromyia bahiensis	Sherlock e Carneiro, 1963

Evandromyia carmelinoi	Pessoa et al 2008
Evandromyia inpai	Alencar 2012
Evandromyia lenti	Sherlock 1957, Pessoa et al 2008
Evandromyia saulensis	Hanson, 1968
Evandromyia tupynambai	Carneiro e Sherlock, 1964
Evandromyia walkeri	Ferro et al 1987
Lutzomyia caballeroi	Ogusuku e Perez 1995
Lutzomyia cruciata	Oca-Aguilar et al 2017
Lutzomyia diabolica	Linquist, 1936
Lutzomyia díspar	Alencar 2012
Lutzomyia gomesi	Mirsa 1952, Hanson 1968
Lutzomyia hartmanni	Hanson, 1968
Lutzomyia lichyi	Hanson, 1968
Lutzomyia longipalpis	Guitton e Sherlock 1969
Lutzomyia noguchii	Ogusuku e Perez 1995
Lutzomyia peruensis	Ogusuku e Perez 1995
Lutzomyia renei	Sherlock, 1957
Lutzomyia sanguinaria	Hanson, 1968
Lutzomyia tejadai	Ogusuku e Perez 1995
Martinsmyia alphabetica	Barreto, 1941
Micropygomyia atroclavata	Carzola 2001
Micropygomyia cayennensis	Hanson, 1968
Micropygomyia chiapanensis	Oca-Aguilar et al 2016
Micropygomyia micropyga	Carneiro e Sherlock, 1964
Micropygomyia oswaldoi	Mangabeira, 1942
Micropygomyia rorotaensis	Alencar 2012
Micropygomyia vextor	Chaniotis e Anderson, 1964
Micropygomyia. Trinidadensis	Hanson 1968, Carzola e Oviedo 2015
Migonemyia gorbitzi	Hanson, 1968
Migonemyia migonei	Barreto, 1941
Notofairchildia stenygros	Alencar et al 2016
Nyssomyia antunesi	Ward, 1976
Nyssomyia hernandezi	Carzola e Oviedo 2001
Nyssomyia intermedia	Barreto, 1941; Bahia et al., 2007
Nyssomyia trapidoi	Hanson, 1968
Nyssomyia whitmani	Barreto, 1941; Bahia et al., 2007
Nyssomyia ylephiletor	Hanson, 1968
Nysspmyia anduzei	Ward, 1976
Nysspmyia umbratilis	Alencar et al 2018
Pintomyia evansi	Carzola et al 2010
Pintomyia fischeri	Barreto, 1941

Pintomyia monticola	Barreto, 1941
Pintomyia ovallesi	Hanson, 1968
Pintomyia pacae	Alencar 2012
Pintomyia pessoai	Barreto, 1941
Pintomyia serrana	Hanson, 1968
Pintomyia verrucarum	Ogusuku e Perez 1995
Pintomyia youngi	Carzola e Oviedo 1998
Pressatia camposi	Hanson, 1968
Pressatia choti	Carneiro e Sherlock, 1964
Pressatia dysponeta	Hanson, 1968
Pressatia triacantha	Mangabeira, 1942
Pressatia trispinosa	Carneiro e Sherlock, 1964
Psathryromyia aclidifera	Hanson, 1968
Psathryromyia dasymera	Hanson, 1968
Psathryromyia geniculata	Hanson, 1968
Psathyromyia brasiliensis	Mangabeira e Sherlock, 1962
Psathyromyia carpenteri	Hanson, 1968
Psathyromyia dendrophyla	Alencar 2012, Oliveira 2013
Psathyromyia lanei	Barreto, 1941
Psathyromyia pascalei	Carneiro e Sherlock, 1964
Psathyromyia runoides	Hanson, 1968
Psathyromyia scaffi	Oliveira 2013
Psathyromyia shannoni	Hanson 1968, Oliveira 2013
Psathyromyia. barretoi	Hanson, 1968
Psychodopygus arthuri	Barreto, 1941
Psychodopygus carrerai carrerai	Hanson, 1968
Psychodopygus davisi	Ward, 1976
Psychodopygus lainsoni	Ward, 1976
Psychodopygus panamensis	Mirsa 1952, Hanson 1968
Psychodopygus paraensis	Ward, 1976
Psychodopygus wellcomei	Ward, 1976
Trichopygomyia longispina	Mangabeira 1942
Trichopygomyia trichopyga	Alencar 2012
Trichopygomyia triramula	Hanson, 1968
Viannamyia furcata	Hanson, 1968

The sand flies *Mi. migonei* (França) is an important vector of *Leishmania (Viannia) braziliensis*, one of the causative agent of cutaneous leishmaniosis in South America, especially in Brazil (Araújo Filho, 1979; Rangel et al., 1990; Queiroz et al.,

1994). Studies verified that this species is also associated with the transmission of *Leishmania infantum chagasi*, in Brazil and Argentina Carvalho et al. (2007). Guimarães et al. (2016) in *Mi. migonei* infection experiments with *Le. in. chagasi* verified that this sandfly species is permissive to the infection of this species of *Leishmania*, demonstrate that *Mi. migonei* can be participating in the cycle of transmission of VL in Brazil in Argentina.

Although its importance, few studies have been done about its immature morphology, especially under scanning electron microscopy (SEM). The immature of *Mi. migonei* had been described by Bareto (1941) when he drew the eggs, larvae, and pupae under light microscopy and gave some slight description. Then, the exochorion of eggs under SEM (Fausto *et al*., 2001; de Almeida *et al*., 2004). Fausto et al., (1998) and Pessoa *et al* (2000) described the larval spiracles and Pessoa *et al*., (2001) described the larval antennae. This paper aims to give more morphological details of immature stages of *Mi. migonei* and provide more information on morphological characters that could be useful for future works on phylogenetics and systematics involving immature stages.

Material and Methods

The immature stages of *Mi. migonei* were obtained from laboratory-reared individuals from progenitors collected in Baturité municipality, Ceará state, Brazil. The species was bred in the Laboratório de Ecologia de Doenças Transmissíveis na Amazônia of the Instituto Leonidas & Maria Deane (ILMD) - Fiocruz Amazônia, according to the method described by Killick-Kendrick & Killick-Kendrick (1991). Some larvae of each larval instar (1^{st} to 4^{th}) and pupae were slide-mounted in a fluid of Berlese. Measurements of the body bristles were made under eye pierce using a light microscopy. Morphology and chaetotaxy of the head were observed following Arrivillaga *et al.* (1999) who pointed morphology and setae of the mouthparts with taxonomical importance. Chaetotaxy of the body followed the system used by Ward (1976a). The chaetotaxy of the pupae used in this study followed the terminology proposed by Oca-Aguilar *et al.*, (2014) Systematic classification follows that proposed by Galati (2003). Also, both species were studied and photographed under a scanning electron microscopy. Some reared larvae were killed in hot water (70^{o}C), fixed in 3% glutaraldehyde and then washed thoroughly in phosphate-buffered saline, the solution being changed every 30 min during six hours. Then, they were then fixed in osmium tetroxide, dehydrated in a series of ethyl alcohol concentrations, submitted to critical point drying in carbon dioxide and spattered with 25 MA colloidal gold. The specimens

were examined with a Scanning electron microscope (JSM5600, JEOL, Tokyo, Japan) and then photographed. Tables were mounted showing the chaetotaxy differences between the instars of each species and between species.

Results

The immatures of *Mi. migonei* described and compared.

The egg of *Mi. migonei*: The egg is elongated, with one side slightly flattened, measuring 323 (300-351) μm in length and 94,8 (89-107) μm in width (N=4) (Fig.1A). The exochorion is formed by a thin basal lamina that supports their ornaments or sculptures with polygonal reticulation, which is comprised by ridges, usually continuous, forming alternating transversal rows of generally rectangular parallel cells or square to polygonal cells (Fig. 1B).

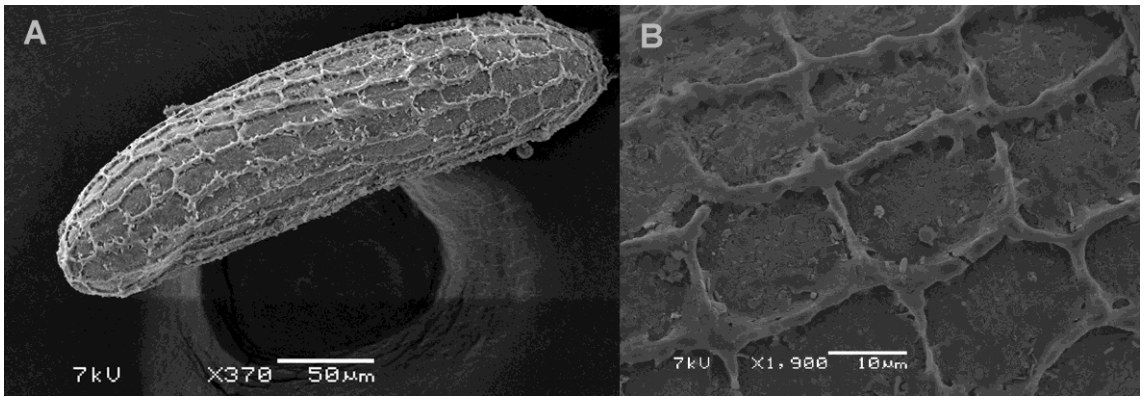

Figure 1. Scanning electron microscopy of the eggs of *Migonemyia migonei*. A General view of the egg showing an ornamentation characterized by the presence ridges arranged in a polygonal pattern. B Eggshell ornamentation showing detail of the ridges.

General appearance of the larvae of *Mi. migonei*: The larva is caterpillar-like, with a well sclerotized hypognathous non-retractile head with very short antennae with short basal tubercle; dark brownish color, head, and body tegument are covered by very small and tiny spines and tubercles scattered distributed. Thorax with a prothorax with the anterior spiracle borne laterally and more two segments, meso and metathorax; and a nine segmented abdomen, covered by brown pale setae and the body tegument yellowish, with a pair of posterior spiracles borne laterally on a short tubercle. The caudal setae are darkened, double-paired the last three instars, simply paired when is in the first instar (Fig. 2A-B). The head is dark brown (Fig. 2A, 3A), body color is pale with darkened eighth and ninth abdominal segments and bears tiny spines in all segments (Fig. 2A, Fig 7). During the first instar is present a prominent egg buster (Fig 3C-D) There are three types of setae, usually distributed in pairs, a barbed brush-like setae, more widely distributed on the larval head and body (Fig.3A, seta 2) and, a little barbed (Fig. 3A, seta 1) and a simple, bare paired setae(Fig. 3A, seta 6). The size and the type of setae are shown in table 2.

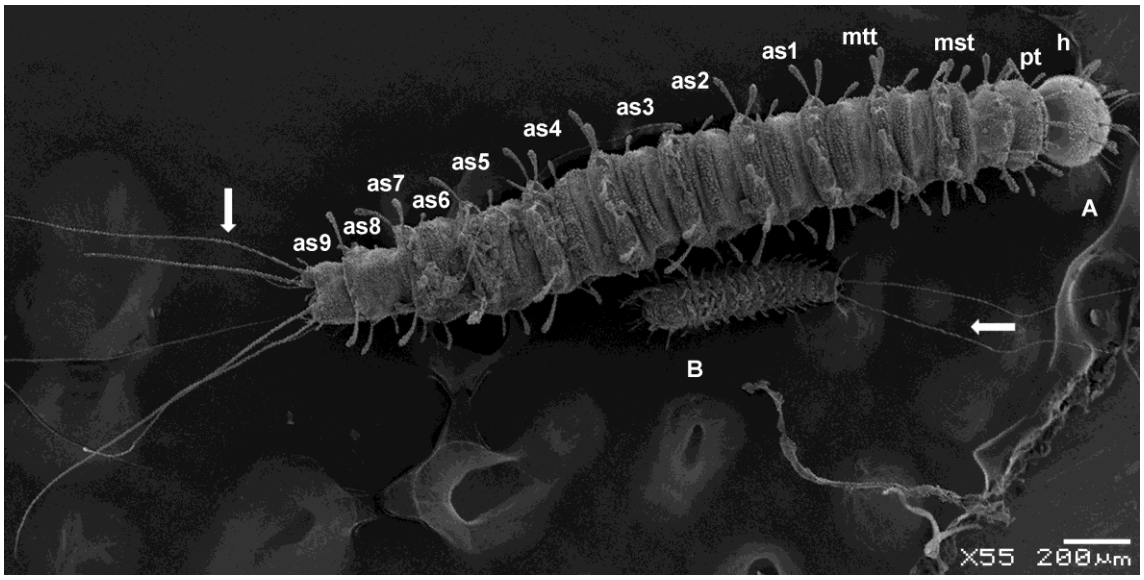

Figura 2. Scanning electron microscopy of the fourth, and first instar larva of *Migonemyia migonei*. A Larva of the fourth instar in dorsal view. B Larva oh de first instar in dorsal view. Caudal Setae.

Head: The head is capsule-like, broader than high. The tegument is covered by thin and small speculation. On the dorsal part of the head (Fig.3A), the cephalic tagma has the following setae: the frontoclipeal anterior setae (1) with spinulate form, the frontoclipeal posterior setae (2) with barbed shape, the genal anterior setae (3) with a simple spine form. The genal medial (4) and genal posterior (5) are barbed brush-like setae. In the ventral part (Fig. 3B), the postgenal (6) and subgenal (7) are simple setae (Table 2). All setae are inserted in small tubercles. In the first instar, the setae 1 is almost simple, however, it is possible to predict the projection of those setae becoming barbed at the other subsequent instars. The antennae of *Mi. migonei* each antenna (Fig. 4A-B) has a basal tubercle (socket), a small and cylindrical segment fused at a second ovoid distal segment. This segment presents an antennal organ equipped with a longitudinal furrow in the posterior surface, more evident in the 1st instar antennae, and three short structures in the base of the segment, the central structure wider than long and shorter than the laterals (Figs. 4A-B).

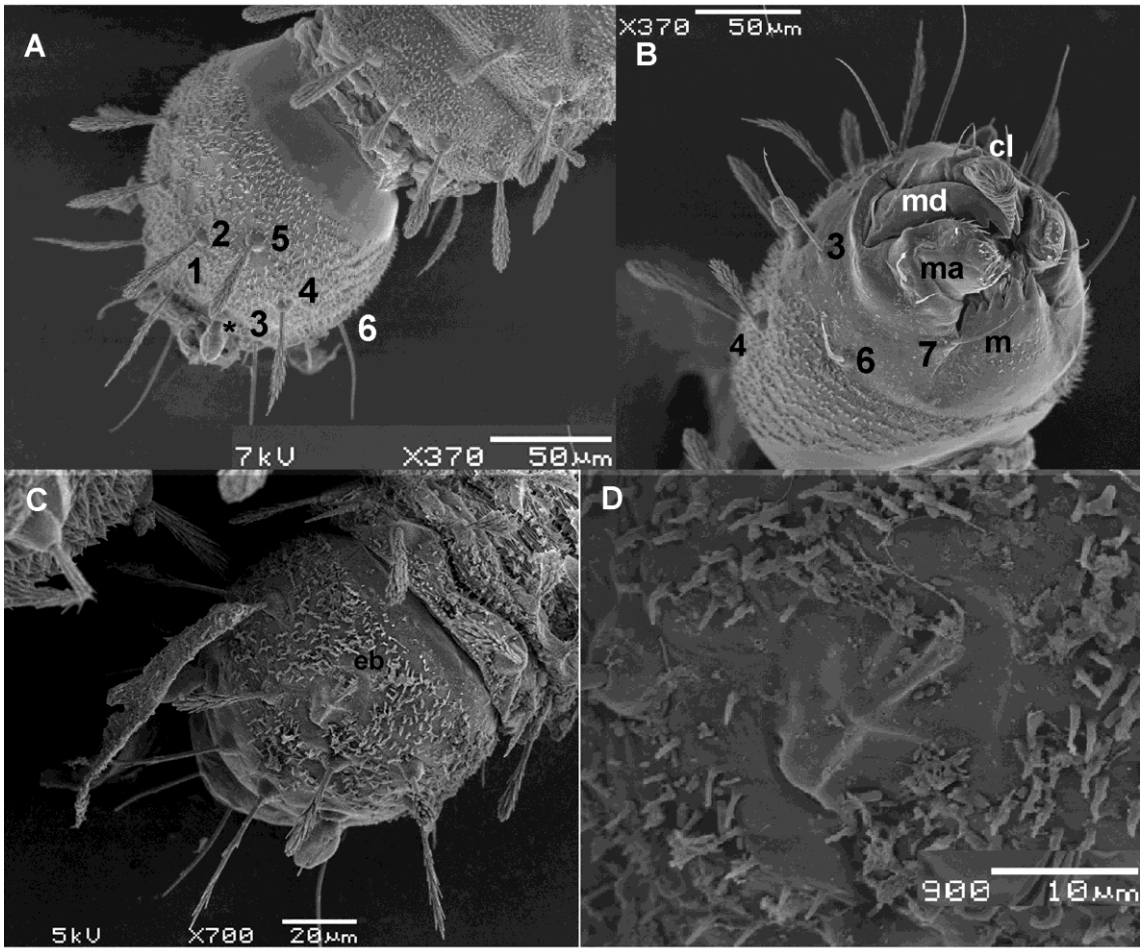

Figure 3. Scanning electron microscopy of the larva of *Migonemyia migonei*. A Head in dorsal of the fourth instar, B and ventral view. C Head in dorsal of the first. D egg buster (eb). cl, Clipeo; md, mandible; ma, maxila; m, mentum Setae numbered according to the chaetotaxy proposed in study.

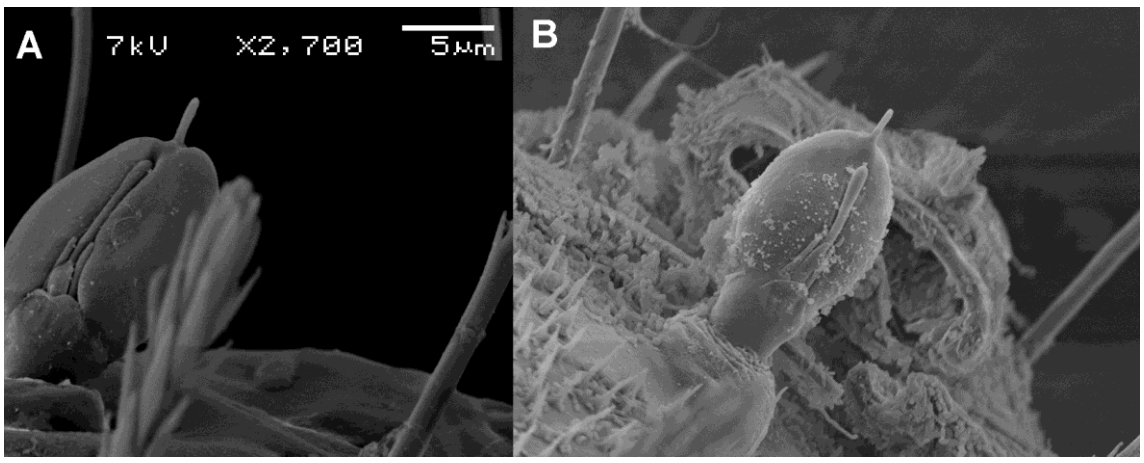

Figure 4. Scanning electron microscopy of the larva of *Migonemyia migonei*. A antenna of the first instar larva. B antenna of the fourth instar larva.

Table 2. Corresponding number and size of the setae of each segment (size in μm, N=4) of fourth to first instar larvae of *Migonemyia migonei*.

Part of the larva body	*Migonemyia migonei*					
	Number of setae	Type of setae	Size of the setae of the correspondent larval instar			
Head		bristle	4th	3rd	2nd	1st
Frontoclipeal anterior	1	spinulate	93,3	77,4	65,4	52,0
Frontoclipeal posterior	2	barbed	85,3	65,3	50,7	29,4
Genal anterior	3	simple	113,0	70,65	60,0	41,4
Genal medial	4	barbed	93,3	64	53,3	44,0
Genal posterior	5	barbed	90,7	62,7	46,7	33,4
Postgenal	6	simple	101,3	64,0	54,7	37,3
Subgenal	7	simple	80,0	37,3	24,0	13,3
Prothorax						
Dorsal internal	1	barbed	85,0	57,3	46,7	38,7
Dorsal intermediate	2	barbed	65,0	41,35	20,0	24,0
Dorsal external	3	barbed	87,5	60,0	49,4	NO
"Shoulder" accessory	a	spine	25,0	17,4	17,7	NO
"Shoulder" accessory	b	barbed	16,0	08,0	08,0	NO
Anterior ventrolateral	4	barbed	100,0	61,3	45,4	32,0
Ventral external	5	barbed	67,5	55,0	49,4	30,7
Ventral internal	6	barbed	100,0	64	53,3	29,3
Dorsal submedian	7	barbed	90,0	49,3	33,4	16,0
Mid – dorsal	8	barbed	100,0	64,0	44,0	18,7
Dorsolateral	9	barbed	95,0	52,0	40,0	26,7
Basal	10	barbed	42,5	22,7	12,0	NO
Post-ventrolateral	11	barbed	96,0	53,35	44,0	30,7
Post-ventral	12	spine	13,3	06,7	06,7	06,7
Mid ventral	13	barbed	72,0	42,6	26,7	12,0
Ventral intermediate	14	barbed	17,3	12,0	NO	02,7
Ventral submedian	15	barbed	38,7	21,35	13,3	08,0
Meso and metathorax						
"Shoulder" accessory	a	spine	14,7	10,7	08,0	06,7
"Shoulder" accessory	b	barbed	17,3	9,35	08,0	NO
Anterior ventrolateral	4	barbed	90,0	52,0	37,4	25,4
Dorsal submedian	7	barbed	142,5	69,35	37,4	20,0
Mid – dorsal	8	barbed	152,5	78,65	46,7	21,4
Dorsolateral	9	barbed	127,5	65,3	42,7	29,3
Basal	10	barbed	25,0	13,3	8,0	NO
Post-ventrolateral	11	barbed	78,8	49,3	36,0	17,4
Post-ventral	12	spine	14,7	9,35	NO	05,3

Mid ventral	13	barbed	77,3	38,65	29,3	14,7
Ventral intermediate	14	barbed	22,7	13,3	08,0	04,0
Ventral submedian	15	barbed	44,0	21,4	14,7	09,4
Abdominal segments 1–7						
Dorsal intermediate	2	barbed	35,0	12,0	5,33	02,7
Anterior ventrolateral	4	barbed	102,5	48,0	34,65	16,0
Dorsal submedian	7	barbed	165,0	76,0	38,70	17,4
Mid – dorsal	8	barbed	180,0	93,3	50,65	20,0
Dorsolateral	9	barbed	165,0	82,65	48,0	41,4
Post-ventrolateral	11	barbed	75,0	44,0	36,0	18,7
Post-ventral	12	barbed	30,0	13,3	10,7	NO
Ventral submedian	15	simple	66,7	37,3	32,0	17,4
"C"-		spine	12,0	9,35	8,00	NO
Abdominal segment 8						
Anterior ventrolateral	4	barbed	77,3	34,7	25,4	NO
Dorsal submedian	7	barbed	46,7	20,0	10,7	10,7
Mid – dorsal	8	barbed	147,15	88,0	57,3	12,0
Dorsolateral	9	barbed	118,8	69,4	45,3	37,3
Post-ventrolateral	11	spinulate	52,0	28,0	25,4	12,0
Post-ventral	12	spine	22,0	12,0	06,7	08,0
Ventral submedian	15	spinulate	57,3	26,7	25,4	9,35
"Should accessory"	A	spine	14,7	06,7	05,4	NO
"Should accessory"	B	spine	29,3	12,0	09,4	05,3
Abdominal segment 9						
Anterior ventrolateral	4	simple	220	152	137,5	92,0
Dorsal submedian	7	simple	95,9	64,0	46,7	34,7
Mid – dorsal	8	barbed	57,3	33,4	24,0	52,0
Dorsolateral	9	barbed	57,3	36,0	24,0	16,0
Post-ventrolateral	11	simple	97,2	48,0	38,7	21,3
Post-ventral	12	simple	48,0	22,7	21,3	9,35
Ventral submedian	15	simple	37,3	21,3	14,7	13,3
Internal caudal	IC		1250	950	720	560
External caudal	EC		1045	625	565	NO

Mouthparts: The external mouth part is composed of a pair of mandibles, a pair of maxillae, labrum, and mentum. Each segmented mandible bears two simple setae in the middle of the dorsal part (S1 and S2), and a simple seta (S6) in the superior margin of the mandible, and are very similar as described by Pessoa *et al.* 2008 (Fig. 5). In the lower part of the mandible, there are three strong apical teeth. Each maxilla has three simple setae, an S1 in the apical dorsal part, and two (S2 and S3) in the proximal part

(Fig. 5). There is a maxillary process in the middle of this structure. In the margin of the dorsal part, it has a sequence of the small and sparse comb of spines, very similar to those found in the maxilla of *Ev. lenti* and *Ev. carmelinoi* (Pessoa *et al*., 2008). In the apex, there are papilliform and trichodea sensillae (spinous hairs). On the upper side, there is a row of small setae. The ventral surface of the labrum is covered with parallel, transverse rows of finger-like combs of setae; the dorsal side has two pairs of very small simple setae. The clypeus has two pairs of simple setae, the distal pair small, the apical bigger (Fig. 5).

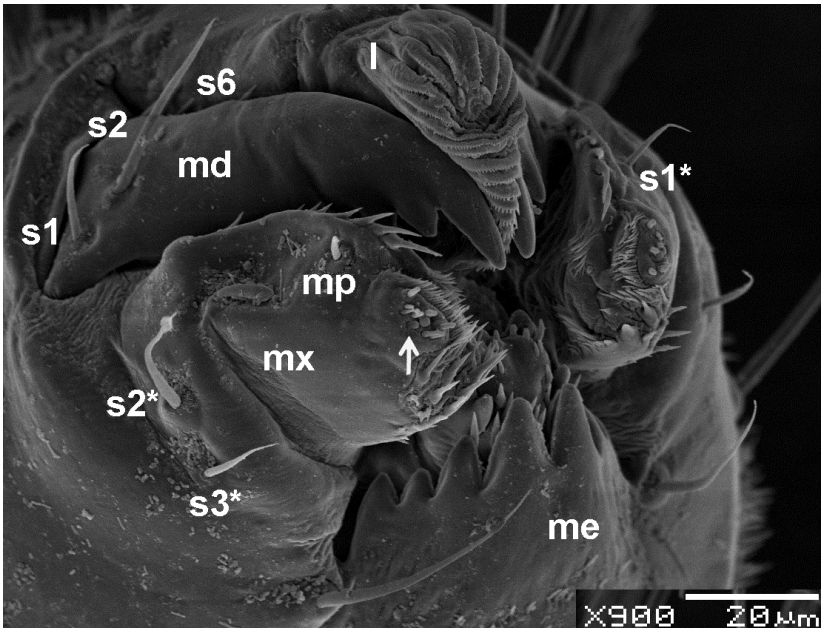

Figure 5. Scanning electron microscopy of the mouthparts of fourt instar larva of *Migonemyia migonei*. L, labrum; md, mandible; mx, maxila; me, mentum; s1-s6, mandible setae; s1*-s3*, maxila setae; satae, maxillary palpus.

The thorax has three segments, the prothorax has the appearance of two segments, the meso and metathorax are homologous with the posterior setae of the prothorax. Chaetotaxy follows the same pattern of setae classified by Ward (1976a) and presented in table 1, a and figure 6 and 7. The anterior spiracles are conical and have eight to nine papillae (Fig. 8 A), five to six to the third instar, 4 to the second (Fig. 8 B). To first instar we did not obtain good images to compare or count.

Chaetotaxy of prothorax: The tergite has two rows of setae, the first row has three pairs of setae, the dorsal internal, dorsal intermediate and dorsal external and the second row with two setae, the dorsal submedian and the mid-dorsal. The pleura has two setae, the anterior ventrolateral and the dorsolateral, that appear to change position in the larva. Those setae are similar, barbed or brushed-like and have just small differences in size (Table 2).

There is a spine hyaline seta, between the first and second rows of setae, usually near the ventrolateral setae. The sternite also has two rows of setae. The first with two similar pairs of setae, a little less barbed than the dorsal setae, the ventral external and the ventral internal. The second row has seven pairs of setae, including the seta b, with different size and shape (Table 2). They are the basal, post-ventrolateral, post-ventral, mid ventral, ventral intermediate, ventral submedian, and b setae. The meso and metathorax do not have the first row of setae of the tergite and sternite of the prothorax and the setae are the same as of the second row of setae of the prothorax.

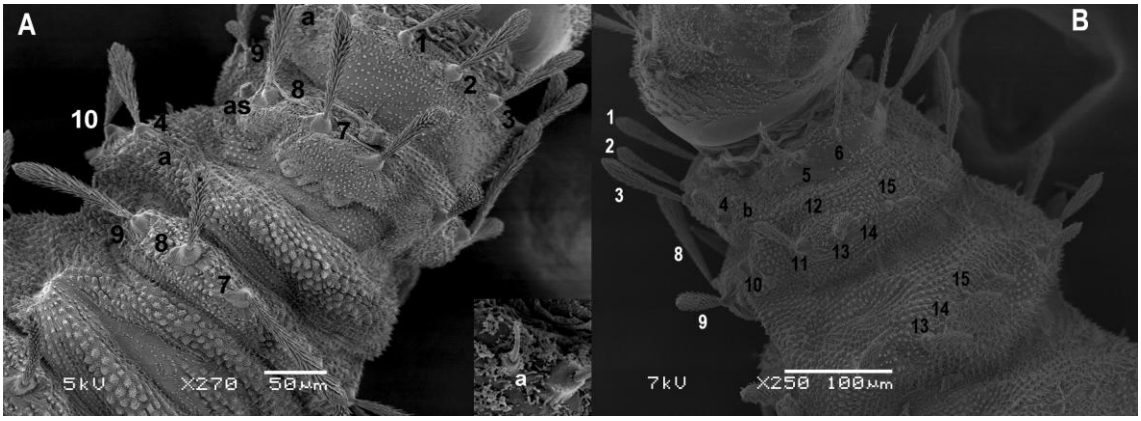

Figure 6. Scanning electron microscopy of the fourth instar larva of *Migonemyia migonei*. A Protorax and Mesotorax in dorsal of the fourth instar, B and ventral view. Setae numbered according to the chaetotaxy proposed in study.

The setae of the abdomen have the same distribution proposed by Ward (1976a), with an absence of the setae 10 in each segment. The segments 1-7 are homologous, with similar size and shape. In the anterior part of the pseudopodium, there is a simple pair of seta, with similar size to setae 11 and 12, not considered by other authors as a taxonomic seta. This seta is called here as "c" (Table 2). The eighth and ninth segments are darker. The posterior spiracles are conical, with 10 papillae. The shape and measurements of the setae are in Fig 7A-B and Table 2. Chaetotaxy of the abdominal segment 1 to 7 is as follows: In the tergite, the pairs of setae are not grouped, anterior dorsal intermediate, much smaller than the others, and the anterior ventrolateral, in the border with the pleura, and a row of pair of setae, dorsal submedian, mid-dorsal and dorsolateral, also in the border of the pleura. All of them are barbed and have different sizes (Table 2). The sternites have large pseudopodia, with a few simple setae, the post-ventrolateral and the post-ventral, both very small simple setae and a simple and large ventral submedian setae. In the anterior part of the pseudopodia, there is a pair of setae, very similar to the post-ventrolateral and the post-ventral, the setae "c". The abdominal segment eight and nine lack pseudopodia. The abdominal segment nine ends in

two tubercles each of which bears a caudal filament (Fig 7 A). The posterior spiracle has 14-11 papillae.

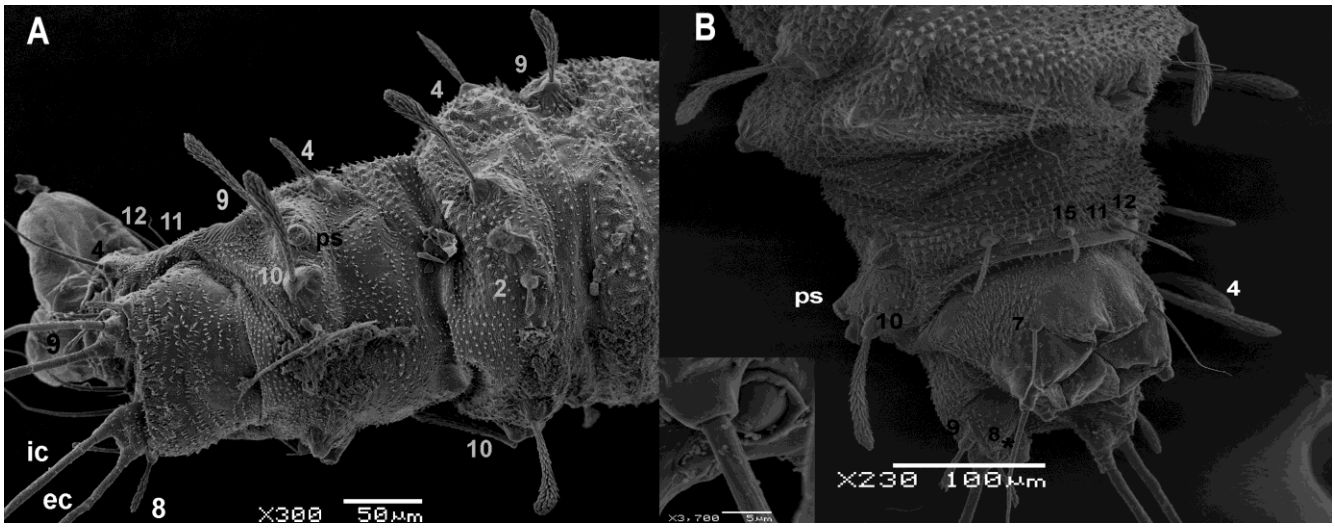

Figure 7. Scanning electron microscopy of the fourth instar larva of *Migonemyia migonei*. A Abdominal 7-9 and in dorsal of the fourth instar, B and ventral view. Setae numbered according to the chaetotaxy proposed in study.

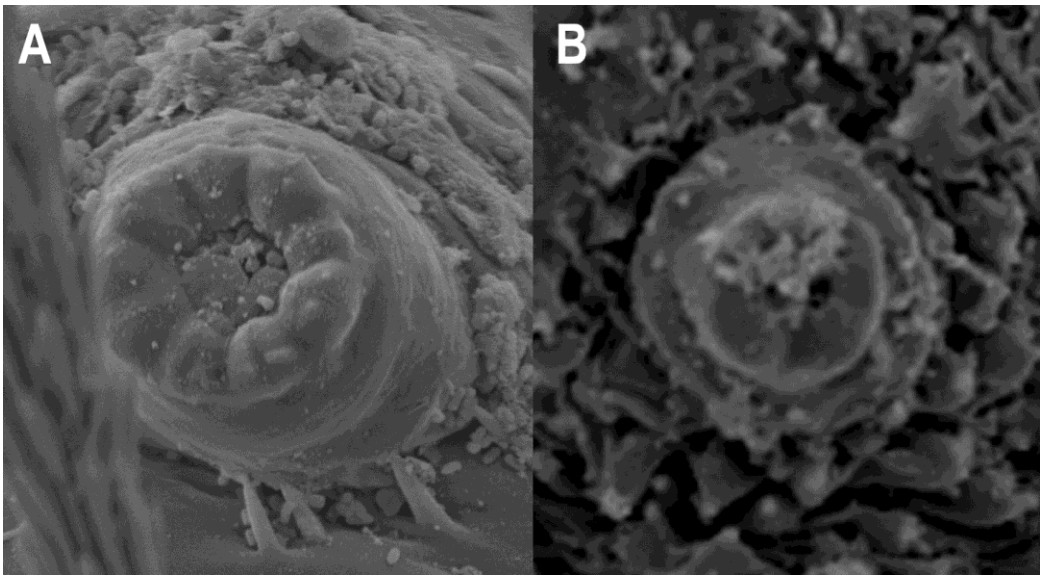

Figure 8. Scanning electron microscopy of the larva of *Migonemyia migonei*. A anterior spiracles of the third instar larva, B anterior spiracles of the second instar larva.

Other larval instars: The body sizes of the instar larvae 3rd to 1st are, from the head to the end of the ninth abdominal segment and with a maximum width of at metathorax, respectively: 1,7 and 0,27; 0,94 and 0,13; 0,56 and 0,09 mm. The first instar is easily identified by the presence of a unique pair of caudal setae (Fig. 2B) and the absence of some bristles in the prothorax (seta 3, a, b and 10) and pro, meso and metathorax (b and 10) and the presence of the egg buster in the head (Fig. 3D). The seta 6 and 14 of the prothorax are

simple in this instar and barbed in the others, the seta 11 and 15 of the abdominal segment 8 are simple and in the other instars become almost barbed. Chaetotaxy for the other instars are the same of the fourth but differ in relation to the size (Table 2).

Pupa description: the pharate Female pupa longer (2.24 mm, n = 5) than pharate males pupa (2.05 mm, n = 5). Head with antennal sheath showing outlines of all flagellomeres of the pre-imaginal stage. Mouthparts sheath smooth; clypeal sheath slightly prominent; head chaetotaxy as presented in Fig 9 and Table 3. Thorax with 10 pairs of setae; prothorax and metathorax with 3 setae in each; mesothorax with four setae, of which two are chaotic prealars, comparatively large (length 0.15 ± 0.008 mm, n = 5) and stout, originated from tubercles and large mesonotal tubercle with continuous border (Fig 10); abdomen with nine visible segments, the width of every segment is near twice its own length, and they diminish gradually in size towards the caudal region (Fig 10 A). Abdominal segments I-II: Tergum with four pairs of setae, pleura, and sternum covered with the thoracic appendage sheaths. Abdominal segment III: tergum and sterna with 4 and 4 pairs of setae, respectively, similar in form and location to the abdominal segments IV-VII; pleura and sterna III partially covered by the thoracic appendage sheaths. Abdominal segments IV-VII: each tergum with 4 setae distributed similarly as the previous segments; each sternum with 4 setae. Abdominal segment VIII: male and female both with two setae pair on the tergo, with two pairs on the sternum and two pairs on the spiracle (Fig 11). All these are very small basiconic setae. Abdominal segment IX: covered by the larval exuvia (as well as VIII), but when uncovered, sexual morphological differences can be observed: in males, there are two lobes at each side, one simple, covering the lateral lobe and the other divided, containing the gonostylus and gonocoxite, and in females two simple and short lobes at each side, one covering the oviscape and the other the cercus; without setae (Fig 11). Abdominal chaetotaxy in Table 3.

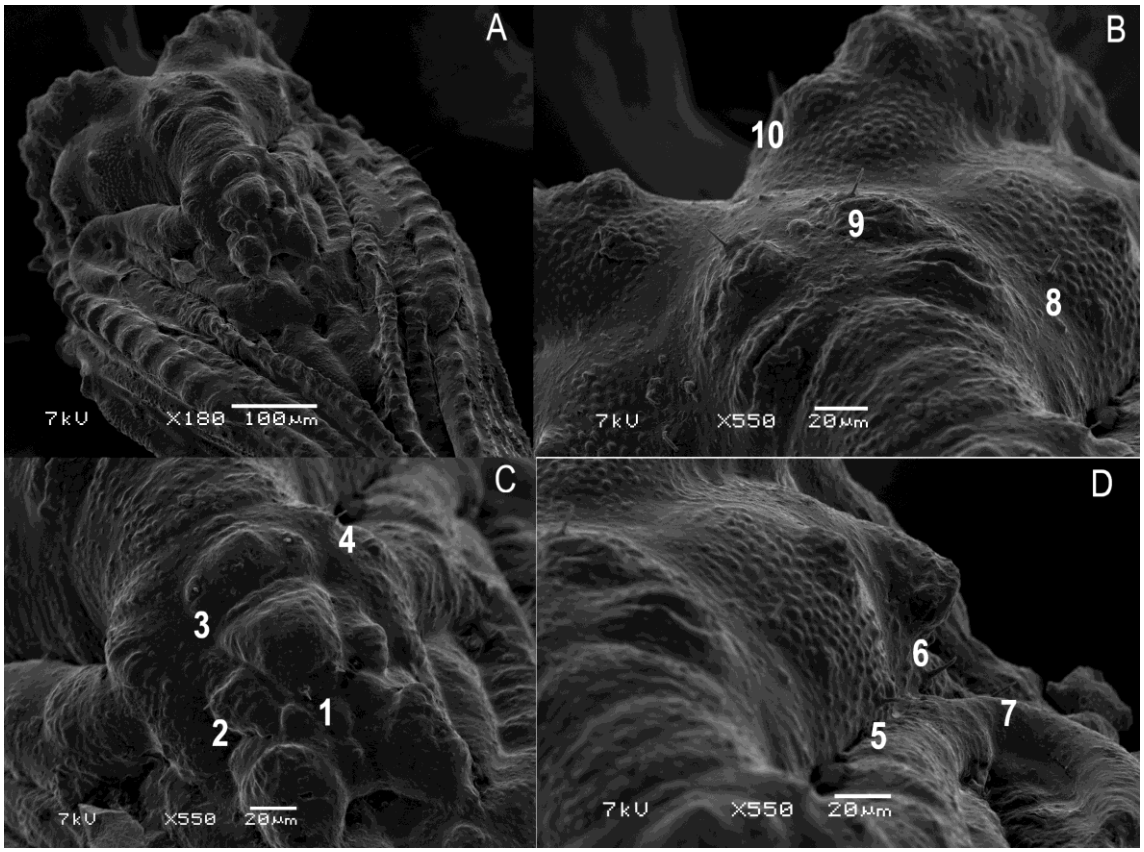

Figure 9. Scanning electron microscopy of the pupa of *Migonemyia migonei*. A head of the pupa. B, C and D, Setae numbered according to the chaetotaxy proposed in study.

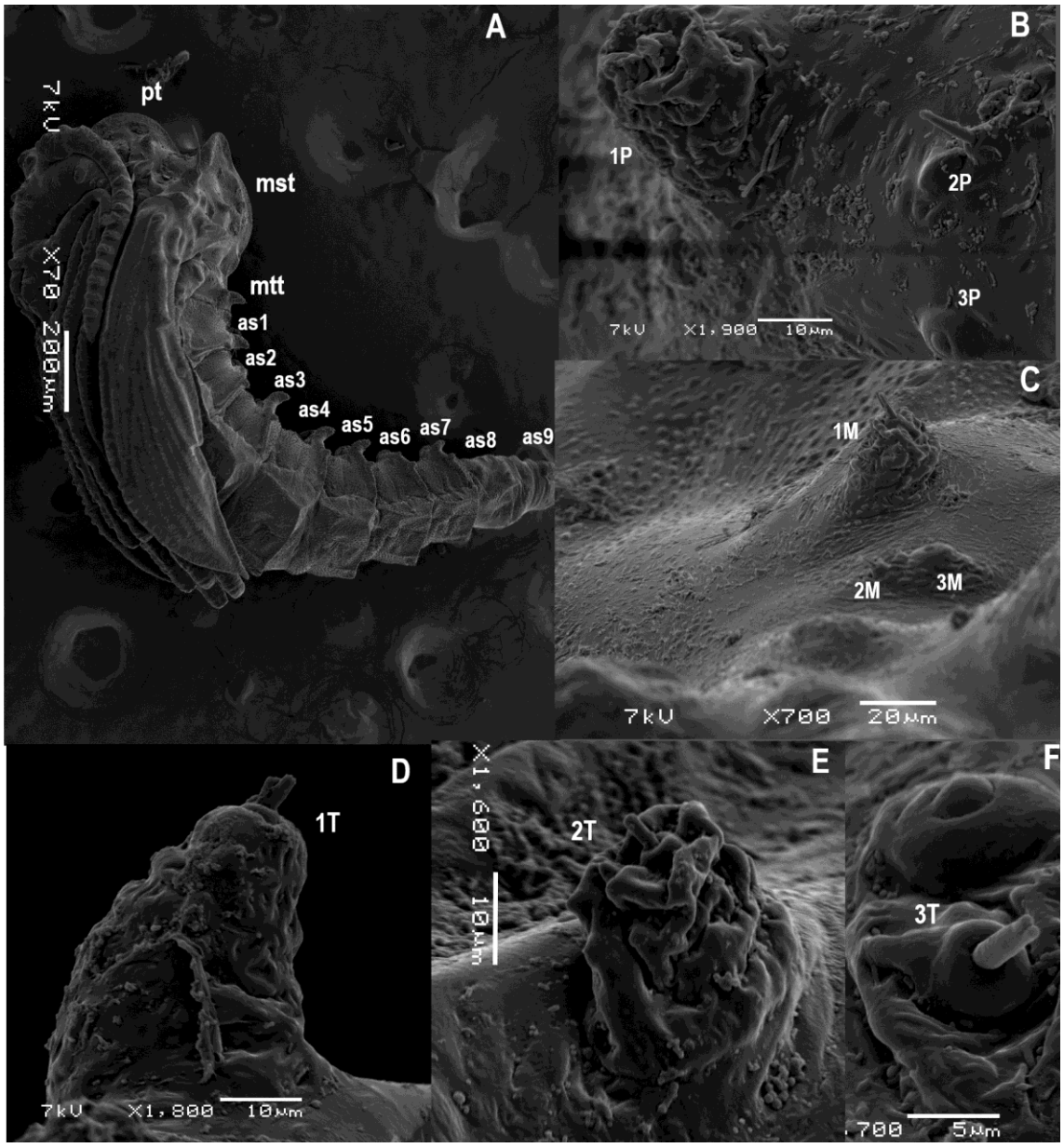

Figura 10. Scanning electron microscopy of the pupa of *Migonemyia migonei*. A Pupa in lateral view. B Prothorax setae, C Mesothorax setae, D, E and F Metathorax setae. Setae numbered according to the chaetotaxy proposed in study.

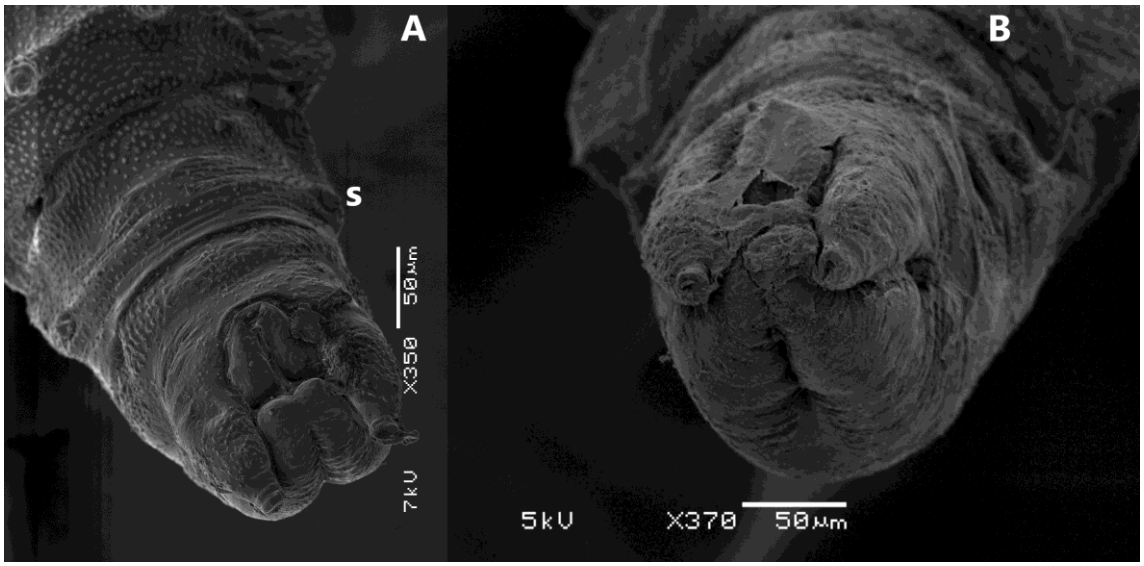

Figura 11. Scanning electron microscopy of the pupa of *Migonemyia migonei*. A Pupa female in ventral view. B Pupa male in ventral view. s, spiracle.

Table 3. Chaetotaxy for pupa of *Migonemyia migonei*.

Tagma	Number	Sensillum type	Terminology
HEAD	1C	Basiconic	Clypeal inferior
	2C	Basiconic	Palpal seta
	3C	Basiconic	Clypeal superior
	4C	Basiconic	Frontal inferior
	9C	Basiconic	Frontal superior
	10C	Basiconic	Vertical
	8C	Basiconic	Frontal medial
	5C	Basiconic	Postocular medial
	6C	Basiconic	Postocular internal
	7C	Basiconic	Postocular external
THORAX			
Prothorax	1P	Basiconic	Protoracic superior
	2P	Basiconic	Prothoracic medial
	3P	Basiconic	Prothoracic inferior
Mesothorax	1M	Styloconic	Mesothoracic inferior
	2M	Styloconic	Mesothoracic medial
	3M	Basiconic	Mesothoracic superior
	4A-B M	Chaetic	Pre-alar
Metathorax	1T	Styloconic	Metathoracic internal
	2T	Styloconic	Metathoracic medial
	3T	Basiconic	Metathoracic external
	5A - B T	Basiconic	Pre-haltere
ABDOMEN			

I-VII	1	Basiconic	Dorsal anterior
	2	Styloconic	Dorsal posterior internal
	3	Styloconic	Dorsal posterior external
	4	Basiconic	Laterodorsal
	8	Basiconic	Ventral posterior internal
	7	Basiconic	Ventral anterior external
	6	Basiconic	Ventral posterior external
	9	Basiconic	Ventral anterior internal
VIII	1	Basiconic	Dorsal superior
	2	Basiconic	Dorsal inferior
	3	Basiconic	Lateral
	4	Basiconic	Lateral
	5	Basiconic	
	6	Basiconic	

Discussion

The exocoronic pattern of *Mi. migonei* is polygonal was first described by Barreto (1941) using only light microscopy and redescribed by Fausto (2001) using SEM. This polygonal pattern of exocorionary sculptures is found in 28 other species of neotropical sandflies (Oca-Aguilar et al 2016).

This polygon pattern being exocorionic to be found in several species of phlebotomine has been questioned whether or not it can be considered as a taxonomic character. Ward and Ready (1975) and Costa et al (2012) suggested that the design could be different according to the environment of the egg deposition site. Perez & Ogosuku (1997) mentioned that the exocoronic pattern does not reflect phylogenetic relationships based on adult characteristics, Pessoa *et al*., (2008) suggested that this pattern may have phylogenetic significance.

The chaetotaxy and morphological structures of phlebotomine larvae, were discussed earlier by several authors (Barretto 1941; Abonnenc, 1956; Hanson, 1968; Ward, 1976a, b; Lane & Sawaf, 1986; Leite & Williams, 1996; Carzola & Oviedo, 2001, 2015; Pessoa *et al*., 2008; Oca-Aguilar *et al*., 2014, 2016, 2017; Alencar *et al*., 2018). The fourth instar larvae of *Mi. migonei* is morphologically similar to *Evandromyia carmelinoi* (Pessoa *et al*., 2008). Despite the similarities, *Mi. migonei*

differs from *Ev. carmelinoi* in some setae, in abdominal segments 1 to 7 setae 11 and 12 are barbed in *Mi. migonei* and simple in *Ev. carmelinoi*. Also occurring in the abdominal segment 8 differences in the same setae, in the species first are almost barbed and in the second are simple.

First instar larvae of sand flies, presents several morphological structures that the other instars do not possess, which are: a pair of caudal bristles, in the jaw, presents four incisive teeth in the other instars are only three and at the apex of the head has a buster of eggs. In addition to these structures, the antenna of the first instar of *M. migonei*, in the distal ovoid track, presents a more evident distal furrow, and three short structures in the base of the segment, the central structure wider than the long and shorter than the laterals. in other instars, this central structure is longer than wider. This morphology of antennas in different larval instars can also be observed in *Lutzomyia longipalpis* (Pessoa et al., 2001), *Nyssomyia intermedia* and *Ny. whitmani* (Bahia *et al*., 2007).

Spiracles of sandflies larvae are inserted apically on bulges, placed laterally in the prothorax and in the eighth abdominal segment (Satchell, 1953), Fausto *et al*., 1998 believe that this arrangement is an adaptation to life in decomposed organic matter. The posterior spiracle of *Mi. migonei* of the present study had 10 papillae, the same as observed by Pessoa *et al*., 2000, whereas Fausto *et al*., 1998 verified that specimens of Venezuela have on average 12.4 papillae, this shows that different regions may be morphologically and phylogenetically different. Costa *et al*., 2018 verified by phylogenetic analysis that adult specimens of *Mi. migonei* from Baturité and Machado form a different clade from Niteroi specimens.

Mi. migonei, its larva IV stage takes on average 13.4 days to empulp (unpublished data), when occurs the metamorphosis the larva releases its exuviae giving rise to pupa. Barreto 1941 described that the abdominal setae 1 of the pupae of *Mi. migonei* that their tip was curly, but the SEM showed that their tip is rounded, the same form of bristles is observed in *Micropygomyia chiapanensis* described by Oca-Aguilar *et al.*, 2016. The pupae of *Mi. migonei* in their eighth follow-up show six setae, a larger number of setae than other Neotropical species, *Dampfomyia beltrani*, *Mi. chiapanensis*, and *Lutzomyia cruciata* present only three sows (Oca-Aguilar *et al*., 2014; 2016; 2017), *Ny. umbratilis* presents a sow (Alencar *et al*., 2018).

Conclusion

In the present study, the immature stages of *Mi. migonei*, an important species of phlebotomine, being considered the main vectors of *Le. braziliensis* in South America and also may be associated with the transmission of *Le. in. chagasi*, were redescribed using SEM. Adding their importance as a vector of these parasites, *Mi. migonei* is suspected to form a species complex, which may show differences in susceptibility as a vector of *Le. braziliensis* and *Le. in. chagasi* (Costa et al., 2018). It is expected that the present description may contribute to the taxonomic of *Mi. migonei*.

Artigo 2

Biology and breeding techniques of *Migonemyia migonei* (Diptera, Psychodidae, Phlebotominae), vector of *Leishmania braziliensis* under laboratory conditions

Biology and breeding techniques of *Migonemyia migonei* (Diptera, Psychodidae, Phlebotominae), vector of *Leishmania braziliensis* under laboratory conditions

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Abstract

Migonemyia migonei is a vector of *Leishmania* in Brazil and has a wide distribution in South America. Phlebotomine colonies allow studies on the biology of the species and their interactions with pathogens, aiming to provide tools for surveillance and control of diseases. The objective of this work is to optimize the colonization conditions of *Mi. migonei* under laboratory conditions. Experiments were conducted to verify the life cycle; fertility and fecundity; longevity, preference of oviposition in a substrate and of a substrate in PVC structure. *Mi. migonei* from egg phase to adult emergence took on average to 62.1 days, with egg stage lasting 10.1 days, larval 37.5 days, and pupal 14.5 days. The mean of oviposited eggs were significantly higher in females fed with Hamster blood (36.68, $p < 0.05$), followed by the human (23.28), mouse (19.63), and chick (12.20). there was no difference between human and mouse blood ($p = 0.1$). Apple-fed females had a significantly longer survival time (10 days, $p < 0.05$) than those fed with sugar water 10% (7 days) and water (5 days). There was no significant difference in the number of eggs laid between the different substrates used ($p > 0.05$). The same is observed in PVC structure ($p = 0.08$). The data indicate that the conditions favouring the development of *Mi. migonei* under laboratory conditions are: supply of apple as a source of sugar and hamster blood as a source of blood mead.

Keywords: life cycle, oviposition, survival

Introduction

Migonemyia migonei is a *Leishmania* (Ross) vector in Brazil, where it presents a wide distribution, occupying various environments, including the Amazon region. This species is a vector of *Leishmania braziliensis*, one of the species that causes american cutaneous leishmaniasis (Araújo Filho 1979, Rangel et al. 1990, Queiroz et al. 1994) and may a putative vector of *Leishmania infantum chagasi* (Guimarães et al 2016).

Some authors verified that this species is also associated with the transmission of *Le. In. chagasi*, in Brazil and Argentina. Carvalho et al. (2007). also verified that in São Vicente Férrer, Pernambuco is an area with visceral leishmaniasis (VL) cases,

where *Mi. migonei* is the second most abundant species. In Fortaleza, Ceará, Silva et al. (2014), suggested that *Mi. migonei* together with *Lutzomyia longipalpis* were *Le. in. chagasi* vectors. Recently, in La Banda, Argentina Salomón et al. (2010) found that in an outbreak of visceral leishmaniasis, *Mi. migonei* could be the putative vector of *Le. in. chagasi*.

Recent studies with *Mi. migonei* in Argentina, Moya et al. (2015) detected DNA of *Le. in. chagasi,* using PCR in specimens collected in the field. In Fortaleza, Rodrigues et al. (2016) detected by qPCR DNA *Le. in. chagasi* in *Mi. migonei*, indicating that it is a potential vector of this *Leishmania* species. Guimarães et al. (2016) in *Mi. migonei* infection experiments with *Le. in. chagasi* verified that this sandfly species is permissive to the infection of this species of *Leishmania* and demonstrated that *Mi. migonei* can be participating in the cycle of transmission of VL in Brazil and Argentina. To such experiments, it is necessary to maintain these sandflies in the laboratory.

Phlebotomine colonies kept in the laboratory are inestimable to understand their biology, physiology, behavior, vector competence, parasite-host interaction, susceptibility or resistance to insecticides and elucidation of taxonomic aspects (Queiroz 1995).

However, to lead out this type of studies it is necessary to maintain stable colonies for several generations, in order to obtain a large number of individuals, sufficient for the execution of the experiments (Killick-Kendrick 1977; El Naiem & Ward 1992). One of the major bottlenecks in colonization maintenance is the low number of individuals per generation, due in large part to the high immature dies and the low fertility rate, which is directly related to the amount of eggs retained due to the death of females before to finish the oviposition (Justiniano et al. 2004, Chagas et al. 2007).

The objective of this research was to evaluate the life cycle of *Mi. migonei* in laboratory conditions, fertility and fecundity with different substrates on oviposition and to verify their longevity with different sources of carbohydrates.

Materials and Methods

Insects

The sand flies used in this study came from the *Migonemyia migonei* colony of the Leônidas and Maria Deane Institute, Fiocruz Amazônia. The colony was in the 62nd

generation, coming from the state of Ceará. This colony is maintained according to the breeding techniques established by Killick-Kendrick 1977.

Groups of three-day-old females were anesthetized on chicks (*Gallus gallus* Linnaeus) for 30 minutes. Females pregnant or engorged with blood were placed for ovulation in breeding pots. These breeding pots measured 7 cm in height, 8 cm in diameter, with a fund replaced with plaster and a leaking cap. The cap was coated with a filó fabric. The pots were kept in plastic boxes in greenhouses B.O.D., with temperature control (26 °C) and humidity (90%). The larvae were fed with rations made from fishes and rabbit food, which were extruded and grounded. The emerged adults were kept in rearing cages.

Life cycle

After three days of blood feeding, the pregnant females were removed from the colony and placed in breeding pots to perform the oviposition. After the females oviposited and died, the laid eggs were transferred to five pots, each with 30 eggs, totalling 150 eggs. Later, the development time from egg hatching to adult emergence was observed. The breeding pots were inspected daily for the removal of fungi and addition of the larval ration. At this stage, to minimize fungal growth, the feed was added only after the first larvae had hatched, using a stereomicroscope. The data were tabulated in Microsoft Excel spreadsheets. This experiment was repeated three times.

Fertility and fecundity

Three-day-old *M. migonei* females were given blood repellent as follows: 20 female chicks (*Gallus gallus* Linnaeus), 20 female hamsters, 20 female mice anesthetized with Ketalar® and 20 females in humans, in breeding cages by one hour in the laboratory at 25 ºC and 95% relative humidity.

After three days, the pregnant females were placed in individual glass tubes with moistened filter paper, the moisture preserved with drops of distilled water. Apple pieces were offered for three days to allow for complete oogenesis and defecation.

At the end of oviposition and death of the females, the eggs were counted and transferred by jets of water to breeding pots. The laying females were dissected under glass slides with the aid of stillets and the retained eggs, if any, were counted and annotated in Microsoft Excel spreadsheet. This experiment was repeated three times.

Substrate for oviposition

After three days of blood feeding, the pregnant females were removed from the colony and placed in breeding pots. In each pot were placed 20 females and 0.3g of

substrate for oviposition, as follows: a pot with rabbit faces, one with apple (*Pyrus malus* L), one with horse faeces, one with debris (faeces of larvae of sandblasted pot), a pot with dead adults and more controls for each substrate, which had cotton with water and cotton with sugary water. The pots were kept in individual plastic cartons in the B.O.D. at 27–29 °C and 70 - 80% RH. This experiment was repeated three times.

Substrate for oviposition in PVC structure

Twenty pregnant females of the colony, three days after the bloodmeal, were placed in a PVC tube structure. The tube, which has 60mm in diameter and 48cm in length, was cut longitudinally and covered by a thin layer of plaster. This gutter was then divided into five sections, on the lower longitudinal axis. In each section, 0.3g of horse faeces, 0.3g of debris, 0.3g of litter, 0.3g of rabbit faeces, and 0.3g of apple were added as a substrate for oviposition. Subsequently, these two axes of the barrel were replaced with the aid of CAPs cast at each end filled with filó. This structure was placed inside a plastic bag where it was kept in a B.O.D. oven at a temperature of 26°C with 80 to 90% humidity. This experiment was repeated three times after the females oviposited and died. The laid eggs were counted with the help of a cell counter, and a stereoscope microscope. Afterward, the dead females were individually dissected and retained eggs were counted, like the laid eggs, were recorded in spreadsheets, being made using the Microsoft Excel program.

Survival

After emergence, 60 females were separated, 20 females for each cage, and for each cage different nutritional sources were offered: apple, 10% sugar water and water (control). Females were recorded for each cage, checked daily and annotated on Microsoft Excel spreadsheets, kept at 27°C and 70-80% of relative humidity at B.O.D.. This experiment was repeated three times.

Statistics

For analysis of the life cycle data, descriptive analyses of minimum, mean, maximum and standard deviation of development were made; for the analysis of fertility and fecundity data, Kruskal –Wallis and Dunn's Multiple Comparison Test. For analysis of the substrate data for oviposition and substrate for oviposition in PVC structure, Variance Analysis (ANOVA) and Tuken; A Mantel-Cox test was used to analyse the longevity data. All tests were done on GraphPad Prism®.

Ethics statement

Sandfly field collections were approved by SisBio (Sistema de Autorização e Informação em Biodiversidade - Permission and Information in Biodiversity System) number 12186. This study was approved by the Brazilian National Ethics Committee (CONEP, 3726) and Ethics Committee on Animal Experimentation, Registration/Protocol: CEUA/UFAM n° 035/2013.

Results

Life cycle

The descriptive data on the developmental time of the immature stages (eggs, larvae, and pupae) of *Mi. migonei* are shown in Table I. The developmental period from egg phase to adult emergence was 62.1 days on the mean. The larval stage 1 (L1) had a duration of 7.9 days, larval stage 2 (L2) 6.8 days, larval stage 3 (L3) 9.4 days, the last larval stage (L4) 13.4 days and the pupal phase 14.5 days (Table 4).

Table 4. Life cycle of *Migonemyia migonei* obtained under laboratory conditions: mean development time in days for each stage.

Stage/Instar	Minimum	Maximum	Mean/Td	SD	N
Eggs/outbreak	6	16	10.1	2.71	450
Instar I	5	12	7.9	2.13	301
Instar II	2	12	6.8	2.81	285
instar III	3	17	9.4	3.79	267
Instar IV	6	24	13.4	4.71	245
Pupal	8	24	14.5	4.20	242
Td			62.1		

Td= total development.

Fertility and fecundity

The fertility of *Mi. migonei* females was higher when fed with hamster blood (2.201), followed by human blood (in 1.397), mouse (1.178) and chick (732). In relation to the fecundity that is the sum of the most retained eggs, the females fed with hamster blood had the highest amount (2.456), followed by human (1.450), mouse (1.348) and chick (1.002; Table 5).

Comparison of *Mi. migonei* fertility data with different blood sources showed significant differences between hamster vs human (p= 0.00062), hamster vs mouse (p= 0.0000015), hamster vs chick (p= 0.000000000000093), human vs pinto (p= 0.000056) and mouse vs chick (p= 0.008) except for human blood vs mouse (p= 0.16; Fig. 12).

Comparison of *Mi. migonei* fecundity data with the different blood sources showed significant differences between hamster vs human (p= 0.0000029), hamster vs mouse (P= 0.000000078), hamster vs chick (p= 0.00000000000000053), human vs chick (p= 0,0016) and mouse vs chicken (P= 0,014) except for human vs mouse (p= 0.4; Fig. 13).

Table 5. Number of eggs laid and retained in *Migonemyia migonei* females fed with the chick, human, hamster and mouse blood under laboratory conditions.

	Chick		Human		Hamster		Mouse	
	Mean ± SD	Total	Mean ± SD	Total	Mean ± SD	Total	Mean ± SD	Total
Laid eggs	12.20±9.59 (0-34)	732	23.28±9.59 (0-50)	1397	36.68±20.13 (0-69)	2201	19.63±14.24 (0-59)	1178
Retained eggs	4.5±8.6 (0-31)	270	0.88±4.93 (0-47)	53	4.25±14.19 (0-70)	255	2.83±8.77 (0-36)	170
Total		1002		1450		2456		1348

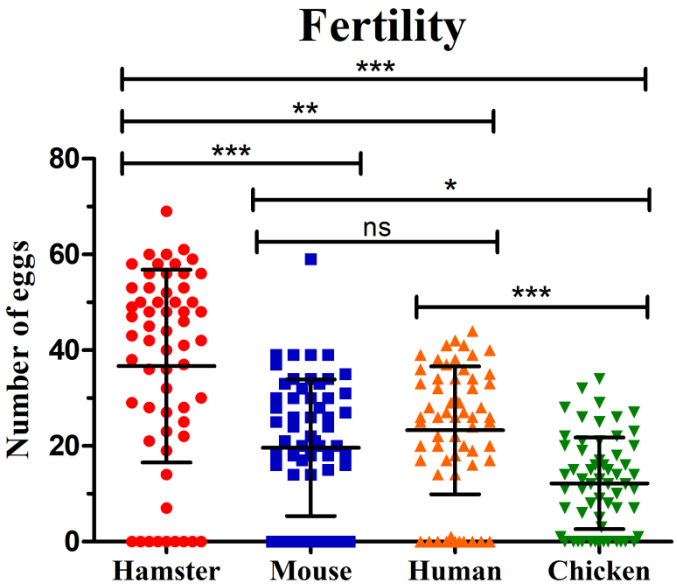

Figure 12. Fertility graph of *Migomemyia migonei* submitted to different blood sources; *= p < 0.005, **= p < 0.005, and ***= p < 0.0005, ns= no significant difference.

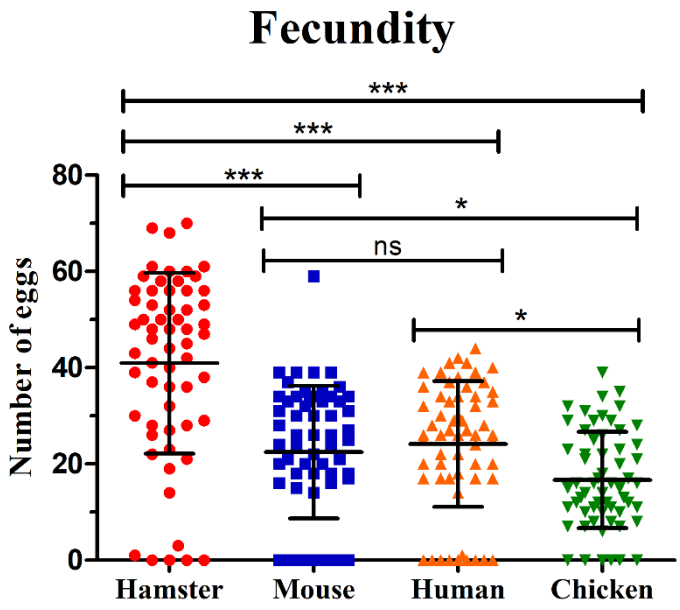

Figure 13. Fecundity graph of *Migonemyia migonei* submitted to different blood sources; *= p < 0.05, **= p< 0.005, and ***= p < 0.0005, ns= no significant difference.

Substrate for oviposition

They were counted in total with apple substrate with 1.225 eggs, followed by horse faeces with 996 eggs, rabbit faeces with 869 eggs, adult died with 869 eggs, litter with 845 eggs and debris with 825 laid eggs (Table 6). The comparison of the eggs of *Mi. migonei* shows that there was no significant difference between the substrates different (p = 0.889) and between the substrates and controls (p = 0.879).

Table 6. Laid eggs by colonized *Migonemyia migonei* females submitted to different substrates for ovulation.

	Post eggs	
Substrates	Mean ± SD	Total
Horse faeces	332±93.92 (224-395)	996
Control 1	267±319.1 (04-622)	801
Control 2	468±207.9 (235-633)	1406
Debris	275±194.6 (84-473)	825
Control 1	249.7±117.5 (132-367)	749
Control 2	377.3±333.2 (32-697)	1132

Litter	281.7±136.2 (131-396)	845
Control 1	392±136.6 (253-526)	1176
Control 2	335.3±173.2 (172-517)	1006
Dead adults	289.7±111.8 (195-413)	869
Control 1	363.3±223.7 (119-558)	1090
Control 2	280± 186.8 (73-436)	840
Apple	408.3±113 (331-538)	1125
Control 1	439.3±174.3(247-587)	1318
Control 2	453.7±112.1 (363-579)	1361
Rabbit feces	323±144 (197-480)	969
Control 1	148±127.3 (1-224)	444
Control 2	237.7±206.2 (51-459)	713

Control 1: cotton with sugar water at 10%; Control 2= cotton with water.

Substrate for oviposition in PVC structure

Were counted in total with horse faeces, substrate with 345 eggs followed apple with 132 eggs, 40 eggs in dead adults, debris with 35 eggs, and litter with 11 eggs laid (Table 7). Comparison of the data with different substrates for oviposition *M. migonei* showed no significant difference between the substrates (p= 0.08661).

Table 7. Eggs laid by *Migonemyia migonei* colonized females, submitted to differences for oviposition in a PVC structure.

	Minimum	Maximum	Mean	SD	Total
Horse faeces	28	174	115	76.9	345
Debris	0	80	48	42.3	144
Litter	0	8	3.6	4.04	11
Dead adults	0	40	13.3	23.0	40
Apple	19	89	44	39.0	132

Survival

The females of *Mi. migonei* presented a longer period of survival compared to the other sources when exposed to the apple sugar source, had a survival median of 10 days, followed by seven days with sugar water and water (control) with 5 days.

Comparison of the survival data of *Mi. migonei* with different sugar sources showed significant differences between apple and sugar water at 10%ada ($p < 0.0001$), apple vs water ($p < 0.0001$), and for water vs sugar water at 10% ($p < 0.0001$; Fig. 14).

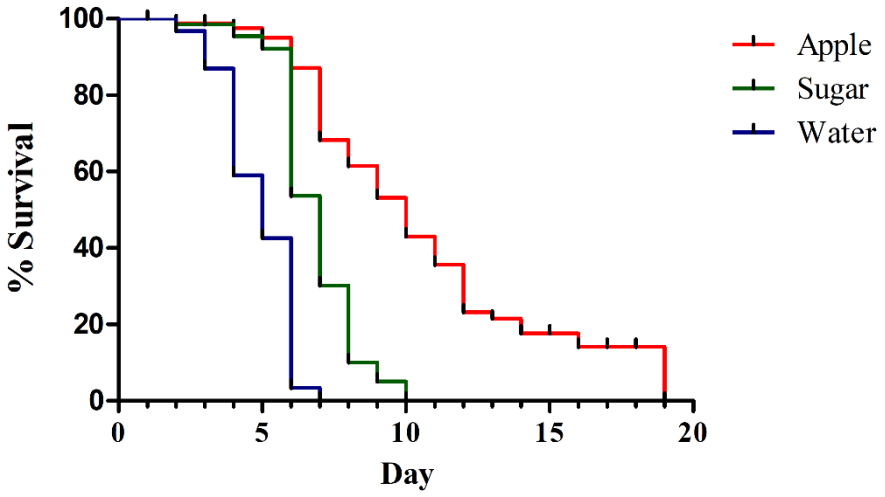

Figure 14. Survival graph of females of *Migonemyia migonei* submitted to different nutritional sources: apple, sugar e water (control).

Discussion

The results present study demonstrated that the life cycle of *M. migonei* lasted 62.1 days on average, from egg phase to adult emergence. These results corroborate Brazil and Brazil (2003), which verified that the life cycle of neotropical sandflies extends for a period of 30 to 100 days, depending on the species considered and the environmental conditions verified during insect development. Ferro et al (1998), verified that the life cycle of a neotropical species, *Lutzomyia shannoni* in the laboratory lasted 54.6 days on average. There are other studies with neotropical species such as *Lutzomyia lenti*, *Tricholateralis cruciata*, and *Nyssomyia umbratilis*, which respectively had a life cycle of 46.8, 52.7 and 75.14 on average (Brazil et al. 1997, Castillo et al. 2015, Justiniano et al. 2004).

In the present study, the average time in days for egg hatching was 11.5 days, while Brazil and Brazil (2003) and Queiroz (1995) found a period of hatching of the eggs, which varies from species to species and incubation temperature. The development of egg to adult insect of phlebotomine can vary from 30 to 40 days,

occurring from 7 to 10 days, the period between oviposition and hatching. The larval period can last for at least three weeks until cramp and from 7 to 14 days for adults to emerge. Rangel and Lainson (2003) found that, on average, eggs of sand flies at 25°C - 27°C start their hatching in 5 to 8 days, but may take more than 15 days to do so.

The fertility experiment demonstrated that hamster blood is the one that produces the highest number of laid eggs compared to the other blood sources studied. Those fed with chick blood showed differences in terms of lower fertility with those fed on blood from human, hamster, and mouse. The nutritional quality of the blood varies among host species and may influence egg yield, rates of development, longevity, and fecundity (Alexander et al. 2002). Chagas et al. (2007) in a similar experiment with *Lutzomyia cruzi* used hamster, human and opossum (*Didelphis marsupialis* Linnaeus), as bloodmeal to this species and found that *Lu. cruzi* fertility was more efficient in hamster blood, human and mucus respectively. Goulart et al. (2017) verified that the best blood source for another neotropical species *Nyssomyia neivai*, is a mouse demonstrating that different species have higher fertility with different blood sources.

In the study with different stimuli for oviposition, the comparative analysis showed no significant difference in females' oviposition. Diverging from the search results of Goulart et al. (2015) with *Ny. neivai* demonstrated that ration (composed of rabbit feces, rabbit ration, fish and ground ration), rabbit faeces and debris attracted adults, and ration was the source of stimulus that best-provided oviposition. Concluding that the largest oviposition was with the richest food source. El Naiem and Ward (1992) observed *L. longipalpis* under laboratory conditions and found that pregnant females exposed to human and rabbit feces increased the number of oviposited eggs, where as Wasserberg and Rowton (2011), who also studied *Lu. longipalpis*, found that debris had a positive influence on the oviposition of the same, contrary to our studies. Kumar et al. (2013) verified the same positivity of oviposition, but with the species *Phlebotomus argentipes*, this may demonstrate a lowering in the maintenance of the *M. migonei* colony.

With these results of oviposition substrates it can be inferred that *M. migonei* are an eclectic species at the time of ovipositing, demonstrated that this species does not have a preference of place to lays its eggs, although some studies verified that it is found related to where they have a large amount of organic matter for their larvae (Brazil et al. 1991, Queiroz et al. 1994). Silva et al. 2014 in a study in Fortaleza

municipality , verified the presence of adults in urbanized areas, where there was the transmission of visceral leishmaniasis.

In the longevity experiment of this work, the best nutritional source offered, compared to other sources, to the females of *Mi. migonei* was the apple. However, Lewis and Domoney (1966) found that several carbohydrates can participate in the nutrition of sand flies, but the sugars most frequently found in different neotropical species are fructose, glucose, and sucrose, corroborating with our work. Fructose is a monosaccharide predominant in several fruits, including apples. In some types of apple, the percentage of fructose is 50% or more (Paganini et al. 2004). The apple used in this work was the Fuji where the sugars are fructose (in greater proportion) sucrose and glucose.

Vegetables may contain 1% to 2% of their weight in the form of free fructose and 3% of fructose in the form of sucrose (Matthews et al. 1987). According to Killick-Kendrick et al. (1977) and Rangel et al. (1985), the use of carbohydrates to feed laboratory sandflies is related to longevity since females fed with sugar solutions had a high rate of survival after oviposition. Chaniotis (1974) suggested that the best sugar food would be sucrose and fructose solutions.

Conclusion

The data indicate that the conditions that favour the development of *Mi. migonei* under laboratory conditions are: supply of apple as a source of sugar and hamster blood as a source of blood meal. These results contribute to the understanding of the mechanisms that influence the breeding conditions in the laboratory of *Mi. migonei* and also how certain dietary factors in adults can affect the biological potential for studies with *Leishmania*.

Artigo 3

Migonemyia migonei is able to transmit *Leishmania infantum chagasi*

Migonemyia migonei is able to transmit *Leishmania infantum chagasi*

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Abstract

Leishmania infantum chagasi is the most widespread etiological agent of visceral leishmaniasis (VL) in the Latin America, with significant mortality rates in humans. This parasite is primarily transmitted by *Lutzomyia longipalpis*, however. the role of *Migonemyia migonei* as a potential vector for this protozoan has been discussed; the parasite is transmitted by the sting of sand flies at the time of blood feeding. Females of *Mi. migonei* created in the laboratory were artificially infected through membrane in mouse blood containing promastigotes of *Le. braziliensis* and *Le. in. chagasi*, dissected 1 and 5 days after infection (PI) and microscopically verified for the presence, intensity and location of *Leishmania* infections. High infection rates of *Le. braziliensis* and *Le in. chagasi* strains were observed in *Mi. migonei*. Metacyclic forms have been found in all vector-parasite combinations since day 5 PI. The parasites were transmitted by bites of infected sand flies to mice. The dose of *Le. in. chagasi* transmitted by a single insect ranged from 10 to 1000 parasites,. Our results point to transmission by *Le. in. chagasi* .

Migonemyia migonei is a vector of *Leishmania braziliensis* in Brazil, where it is widely distributed. In a recent study Guimarães et al. (2016) found that *Mi. migonei* was infected with *Leshmania infantum chagasi*, that therefore this species of phlebotomine is permissive to the infection of this species of *Leishmania*.

Lutzomyia longipalpis is the main vector of *Le. in. chagasi* in South America, but *Mi. migonei* has been found in high abundance in areas of transmission of visceral leishmaniasis. in. Some authors believe that the epidemiology of visceral leishmaniasis is changing, as this species may be a vector of *L. in. chagasi*, together with *Lu. longipalpis*. Moya et al. (2015) in a study in Argentina, verified that specimens of *Mi. migonei* collected in the field, had presence of DNA *Le. in. chagasi.* In Fortaleza, Rodrigues et al. (2016) detected by qPCR DNA *Le. in chagasi* in *Mi. migonei*, indicating that it is a potential vector of this species of *Leishmania*.

Considering these studies demonstrated above, this study aims to develop model of transmission *Le. in. chagasi* through the bite of *Mi. migonei* to a vertebrate.

Females of *Mi. migonei* colonized at the Leônidas and Maria Deane Institute (Marialva artigo 2) were infected by feeding through a chick skin membrane (feeding device) on mouse blood containing 4×10^7/mL *Le. in. chagasi* (MHOM/BR/70/BH46), and *Le. braziliensis* (HMOM/BR/75/H2903, control) promastigotes.

After one, five days of the experiments, the midguts of infected flies were dissected and the parasite load was estimated by hemocytometer counting.

For experimental transmission by bite, five-day infected flies were transferred to small plastic vials (3-dram volume, 4.8-cm height, 1.8-cm diameter) covered at one end with a 0.25-mm nylon mesh. Balb/C mice were anesthetized by Thiopental injection. Clamps were used to hold the mesh end of each vial flat against the animals' ears so that the fly had access to the ear skin for feeding over a period of 1-2 h in the dark. The animals were then euthanized and the exposed ears were dissected for testing the parasite presence. Ten infected sandfly was used for each of the transmission experiments (Figure 15). All animals were maintained at the Animal Care Facility of the FIOCRUZ-MG under specific pathogen-free conditions and were used in accordance to a study protocol approved by the FIOCRUZ Ethical Committee for Animal Use (CEUA; license number LW30/10).

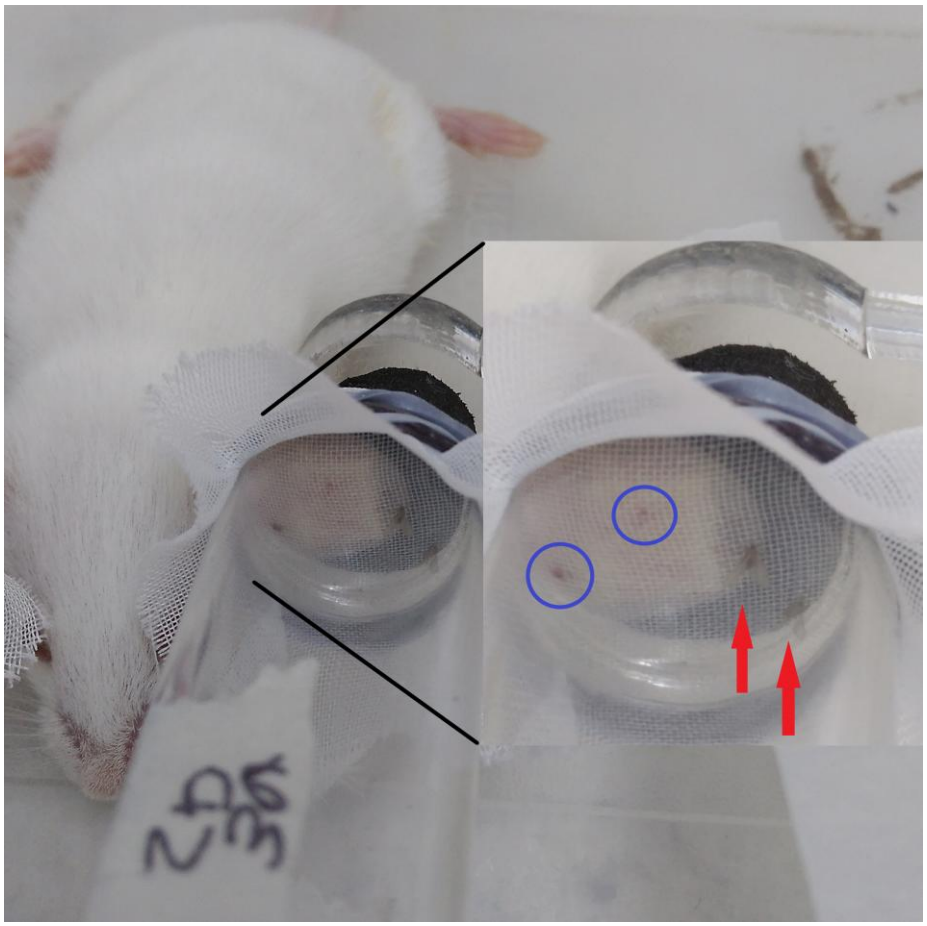

Figure 15. *Migonemyia migonei* sandfly biting a Balb/C mouse. Several sandflies were confined within a vial and allowed to bite the animal's entire ear. Note the blood engorgement of the sand flies (arrow).Inset: Showing the bite site as a small red dot (circle).

Exposed-bite animal ears were processed for DNA extraction and real-time PCR as described by Kimblin et al 2008. Parasite quantification was performed by automatic comparison with the specific set of standard samples prepared in parallel to each set of test samples. The number of *Leishmania* in each sample was summarized as the mean of the two median values from the three reactions in each run. Fourteen ears from each group *Le. in. chagasi* and *Le. braziliensis* were processed.

A total of 300 *Mi. migonei* females from 400 individuals ingested the infected blood meal containing *Le. in. chagasi* promastigotes, and 300 from 400 females for *Le. braziliensis*. To develop a transmission model for laboratory use, we use mice. Twenty-eight ears were exposed, half for 148 females of *Mi. migonei* with *Le. in. chagasi* and the other half for 121 females with *Le. braziliensis*. The detection and quantification of *Le. in. chagasi* transmitted by sandfly bites were performed by real-time PCR. The parasite doses of *Le. in. chagasi* expelled from the sandflies to the ears ranged from 10 to 1000, and for *Le. braziliensis* there was no transmission to the exposed ears (Fig. 16).

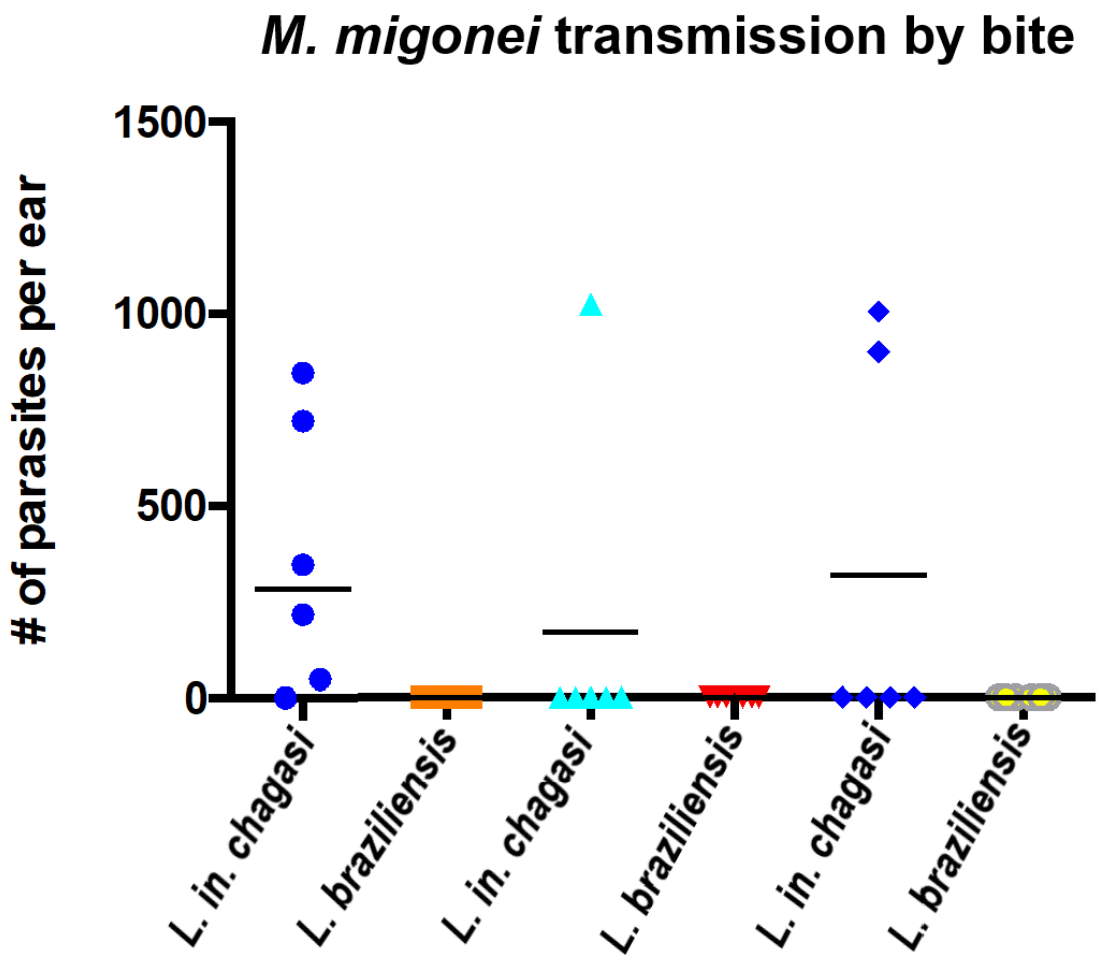

Figure 16. *Leshmania. infantum chagasi* and *Leshmania braziliensis* transmitted to the mouse ear. The parasite numbers were obtained by real-time PCR of dissected ears at two hours after the bites.

It is the first time that *a Leishmania* transmission model in the laboratory uses the *Mi. migonei* species, since other models use only *Lu. longipalpis* for the Americas.

Our results showed that the number of promastigotes of *Le. in. chagasi* transmitted by *Mi. migonei* to the ears ranged from 4 to 1,000 (Fig 16). Secundino et al., 2012 obtained similar numbers with *Lu. longipalpis*, demonstrating that *Mi. migonei* is a competent vector in the transmission of *Le. in. chagasi* Kimblin et al. 2008 using Old World species, *Phlebotomus duboscqui*, was able to transmit up to 100,000 *Leishmania major* parasites, but most of the insects transmitted up to 600 parasites.

These numbers of parasites transmitted by *Mi. migonei* may even increase if we had offered a second blood meal, it was demonstrated by Serafim et al 2018 that *Lu. longipalpis* and *Ph. papatasi* infected with *Le. in. chagasi* and *L. major* respectively,

after a second repast the parasite load of the leishmanias in the vectors had an increase of 4.5 times.

The transmission of *Le. braziliensis* may not have occurred due to the low virulence of the strain, the time of *leishmania* growth in the vector (Fig 16), was not enough to migrate from the same to the thoracic region where the mitral valve is located. Nieves & Pimenta 2000 verified that the amount of *Le. braziliensis* in *Mi. migonei* up to the fifth day in the thoracic region was less than 10%.

We conclude that *Mi. migonei* is able to transmit *Le. in. chagasi*, this proves that phlebotomine is playing a fundamental role in the epidemiology of visceral leishmaniasis in Brazil and Argentina.

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