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Lucas Christian de Sousa Paula

Avaliação da bionomia, genética e compatibilidade reprodutiva de três populações de *Lutzomyia longipalpis* sensu lato

Recife
2022

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Tese apresentada ao Doutorado em Biociências e Biotecnologia em Saúde, do Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, para obtenção do grau de Doutor em Ciências.

Orientador: Dr. Filipe Dantas Torres

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“A princípio só se admitia uma espécie (...),
hoje, porém, é fato certo e confirmado que
existem várias.”
Lutz e Neiva (1912).

RESUMO

PAULA, Lucas Christian de Sousa. **Avaliação da bionomia, genética e compatibilidade reprodutiva de três populações de *Lutzomyia longipalpis* sensu lato**. 2022. Tese (Doutorado em Biociências e Biotecnologia em Saúde) – Instituto Aggeu Magalhães, Fundação Oswaldo Cruz, Recife, 2022.

A leishmaniose visceral é uma doença negligenciada que representa um grande problema de saúde pública em áreas endêmicas. A doença é causada pelo protozoário *Leishmania infantum* e o flebotomíneo *Lutzomyia longipalpis* é o seu principal vetor nas Américas. Entretanto diversos estudos têm indicado que se diferentes populações de *L. longipalpis* podem representar diferentes táxons sob a espécie nominal *L*. *longipalpis*. Um parâmetro avaliado, por exemplo, é o fenótipo de populações nas quais os machos podem apresentar um par de manchas dorsolaterais no quarto tergito abdominal ou dois pares no terceiro e quarto tergitos, conhecidos como fenótipos 1S e 2S, respectivamente. Na década de 1980 estudos de cruzamentos mostraram a presença de isolamento reprodutivo entre populações de localidades e fenótipos diferentes do Brasil. Outros indícios também reforçam a hipótese da existência de várias espécies crípticas que são referidas como *L*. *longipalpis* sensu lato, apesar do número de membros ainda ser incerto. O objetivo deste estudo, foi comparar a bionomia de populações de *L*. *longipalpis* s.l. mantidas em laboratório, bem como foi avaliar a estrutura genética e a compatibilidade reprodutiva dessas populações. Para tal, três colônias de *L*. *longipalpis* s.l. oriundas de três estados brasileiros: Passira (fenótipo 2S, PE), Santarém (fenótipo 1S, PA) e Teresina (fenótipo 1S, PI) foram estabelecidas em laboratório. A estrutura genética e a relação filogenética das três populações foram avaliadas utilizando um fragmento do gene *period* como marcador. Os dados obtidos revelaram a presença de dois clados bem definidos, destacando a população de Teresina 1S como diferente das populações de Passira 2S e Santarém 1S, as quais foram identificadas como sendo do mesmo grupo genético. Além disso, os dados genéticos indicaram um fluxo gênico limitado entre as populações dos dois clados corroborando a estrutura genética encontrada. Em seguida, os cruzamentos, visando a fim de identificar a existência de barreiras reprodutivas entre as três populações, refletiram os dados genéticos, demonstrando uma barreira reprodutiva entre a população de Teresina 1S quando comparada àquelas de Passira 2S e Santarém. Os ciclos de vida das proles produzidas até a emergência imaginal também foram analisados. Os resultados reforçam a hipótese da existência de diferentes espécies em *L*. *longipalpis* s.l., com membros geneticamente distintos e reprodutivamente isolados. O conjunto de dados demonstram a necessidade de descrição formal dessas espécies crípticas e são úteis para esta finalidade. A identificação das espécies é um passo crucial para a caraterização de atributos importantes tais como suscetibilidade a patógenos, a inseticidas e outros associados.

Palavras-chave: genética populacional; Psychodidae – genética; Psychodidae - classificação; acasalamento cruzado; filogenia; vetores de doenças.

ABSTRACT

PAULA, Lucas Christian de Sousa. **Evaluation of the bionomy, genetics and reproduction compatibility of three *Lutzomyia longipalpis* sensu lato populations**. 2022. Thesis (Doctorate in Biociências e Biotecnologia em Saúde) – Aggeu Magalhães Institute, Oswaldo Cruz Foundation, Recife, 2022.

Visceral leishmaniasis is a neglected disease that represents a great public health problem in endemic areas. This disease is caused by the protozoa parasite *Leishmania infantum* and the phlebotomine sand fly *Lutzomyia longipalpis* is its main vector in the Americas. However, several studies have been pointed out different populations may represent different species under the nominal species *L*. *longipalpis*. For instance, a trait observed is male populations of *L. longipalpis* can present two phenotypes: one pair of whitish pale spots on fourth abdominal tergites, or two pair of spots on the third and fourth tergites, namely 1S and 2S phenotypes, respectively. In the 1980, crossbreeding studies showed a reproductive isolation between different Brazilian populations with distinct spot-phenotypes. Since then, there has been growing interest on the taxonomic status of *L. longipalpis*. A couple of studies underpins the existence of several cryptic species that collectively are referred to *L. longipalpis* sensu lato, although the number of species remain uncertain. The goal of the present study was comparing the bionomy of three laboratory-reared populations of *L. longipalpis* s.l., as well as evaluated their genetic structure and the reproductive compatibility. For this propose, three colonies of *L. longipalpis* s.l. were established with individuals originated from three Brazilian states: Passira (2S phenotype; Passira, Pernambuco), Santarém (1S phenotype; Santarém, Pará) e Teresina (1S phenotype; Teresina, Piauí). Genetic structure and phylogenetic relationship were assessed using a fragment of the period gene as a marker. The genetic data disclosed the presence of two well-supported clades, highlighting Teresina (clade I) as a different group when compared to Passira and Santarém populations (clade II), whose were assessed as same genetic group. In addition, genetic differentiation data suggested a limited gene flow between the populations of the clade I and clade II. Thereafter, crossbreeding experiments, were carried out for testing the reproductive compatibility between the three populations. The results showed a partial reproductive isolation between the population of Teresina when compared to Passira and Santarém, where no difference was observed between the two later. The developmental cycles of the offspring were also evaluated. The results presented herein underpin the existence of different species in *L. longipalpis* s.l., which are genetically different and show reproductive barriers. This study, altogether with previous ones, will serve as basis for the formal recognition and description of the cryptic species of *L. longipalpis* s.l.

Keywords: population genetics; Psychodidae – genetics; Psychodidae – classification; Hybridization; phylogeny; disease vectors.

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1 INTRODUÇÃO

Os flebotomíneos são insetos de grande importância médico-veterinária, por serem capazes de transmitir vários agentes patogênicos aos animais e seres humanos (READY, 2013). Em particular, eles são os vetores biológicos de protozoários do gênero *Leishmania*, responsáveis por causar doenças com considerável morbidade e mortalidade em 98 países e territórios no mundo (ALVAR *et al*., 2012). A leishmaniose visceral, forma mais grave da doença, é endêmica em 71 países e o Brasil é o principal foco nas Américas (ALVAR *et al*., 2012; SOUSA-PAULA; OTRANTO; DANTAS-TORRES, 2020).

Quando Adolpho Lutz e Artur Neiva (1912) descreveram a espécie *Lutzomyia longipalpis* (referida a partir daqui como *L. longipalpis sensu stricto*), dificilmente eles poderiam imaginar que estariam descrevendo um dos insetos vetores de maior importância médico-veterinária nas Américas. Tampouco, que essa espécie de flebotomíneo estaria envolvida em uma discussão sobre o seu status taxonômico que vem perdurando a mais de meio século.

Desde as observações por Mangabeira Filho (1969), várias populações de flebotomíneos identificados morfologicamente como *L. longipalpis* (referidos a partir daqui como *L. longipalpis* sensu lato) do Brasil e outros países neotropicais têm sido avaliadas a fim de entender a história evolutiva e elucidar os problemas taxonômicos envolvendo a espécie originalmente descrita por Lutz e Neiva (SOUZA; BRAZIL; ARAKI, 2017). Em particular, vários estudos têm utilizado marcadores genéticos para avaliar as populações de *L*. *longipalpis* s.l. (BAUZER *et al*., 2002a, 2002b; ARAKI *et al*., 2009, 2013; Lima Costa *et al*., 2015). Outros estudos têm utilizado outras características, como os sons copulatórios (*love songs*), a fim de diferenciar essas populações (ARAKI et al., 2009; VIGODER et al., 2015).

As diferenças genéticas e de sons copulatórios têm reforçado a hipótese de isolamento reprodutivo entre certas populações de *L*. *longipalpis* s.l. e, consequentemente, fortalecido hipótese da existência de diferentes espécies crípticas (ARAKI *et* al., 2009, 2013; LANZARO et al., 1993; SOUZA *et* al., 2008; WARD *et al*., 1983). Porém, poucos estudos têm explorado cruzamentos interespecíficos entre flebotomíneos até então (DOS REIS; ALEVI, 2020). Embora outras espécies válidas (*i.e*., *L*. *alencari*, *L*. *cruzi*, *L*. *gaminarai*, *L*. *matiasi*, *L*. *pseudolongipalpis*) também compõem o complexo *L*. *longipalpis* (SOUSA-PAULA et al., 2021b), apenas um único

estudo avaliou hibridização entre *L*. *longipalpis* s.l. e outra espécie nominal do complexo (ARRIVILLAGA; SALERNO; RANGEL, 2009). No estudo em questão, os autores reportaram um isolamento reprodutivo assimétrico entre populações venezuelanas de *L*. *longiplapis* s.l. e *L*. *pseudolongipalpis*. A espécie *L*. *pseudolongipalpis* é filogeneticamente próxima a *L*. *longipalpis* s.l., sendo suas fêmeas distinguíveis morfologicamente, enquanto os machos são idênticos (GALATI, 2018). Lanzaro *et al*. (1993) reportaram que híbridos entre populações de *L*. *longipalpis* s.l. do Brasil (Lapinha-MG), Colômbia e Costa Rica poderiam ser estéreis, devido a presença de anomalias morfológicas dos espermatozoides. Se os híbridos são capazes de manter populações próprias ao longo de gerações, é algo que precisa ser ainda compreendido, principalmente na natureza.

O interesse taxonômico sobre *L*. *longipalpis* s.l. tem crescido ao longo das últimas décadas (SOUZA *et al*., 2017). Até meados dos anos 2000, muitos autores questionavam a hipótese da existência de diferentes espécies crípticas entras as populações de *L*. *longipalpis* s.l. (MUKHOPADHYAY *et al.*, 1998a, 1998b; MUTEBI *et al.*, 1998; RANGEL *et al.*, 1999; DE QUEIROZ BALBINO *et al.*, 2006; AZEVEDO *et al.*, 2008). Esses autores defendiam que *L*. *longipalpis* s.s. seria uma única espécie, porém altamente polimórfica. Contudo, atualmente as diferentes linhas de evidências predispõe consenso sobre a existência de várias espécies.

Uma pergunta que tem recebido pouca atenção é: Quem seria, dentre as várias populações de *L*. *longipalpis* s.l. conhecidas, a espécie originalmente descrita em Lutz e Neiva em 1912? Atualmente, essa é uma pergunta sem resposta, considerando que os espécimes utilizados na descrição original não se encontram disponíveis (BRANDÃO-FILHO et al., 2009; SOUSA-PAULA; DANTAS-TORRES, 2021). Na ausência de um holótipo disponível, a designação de um neótipo para *L*. *longipalpis* s.s. se faz necessária e imprescindível para solucionar os problemas taxonômicos que envolvem essa espécie tão importante (SOUSA-PAULA; DANTAS-TORRES, 2021). Além da redescrição morfológica, esse ato taxonômico deve ser realizado em conjunto com a caracaterização de marcados moleculares (mitocondriais, nucleares ou ambos) e, possivelmente, sons copulatórios e feromônios sexuais. Após a fixação do neótipo, será possível comparar quais populações de *L*. *longiplapis* s.l. diferem da espécie verdadeira (*senso stricto*).

Em vista da discussão taxonômica que perdura, o presente estudo avaliou geneticamente três populações de *L. longipalpis* s.l. Também foi avaliada a

compatibilidade biológica através de cruzamentos intra e interpopulacionais e o desenvolvimento do ciclo de vida dessas populações. Nas seções a seguir, serão revisados os principais aspectos relacionados aos flebotomíneos, desde a biologia até a importância desses insetos como vetores de patógenos, com ênfase nos agentes causadores das leishmanioses, e serão revisados estudos dedicados ao complexo *L. longipalpis* s.l. Por fim, são apresentados na íntegra três artigos publicados e um manuscrito com os principais resultados obtidos.

2 REVISÃO DA LITERATURA

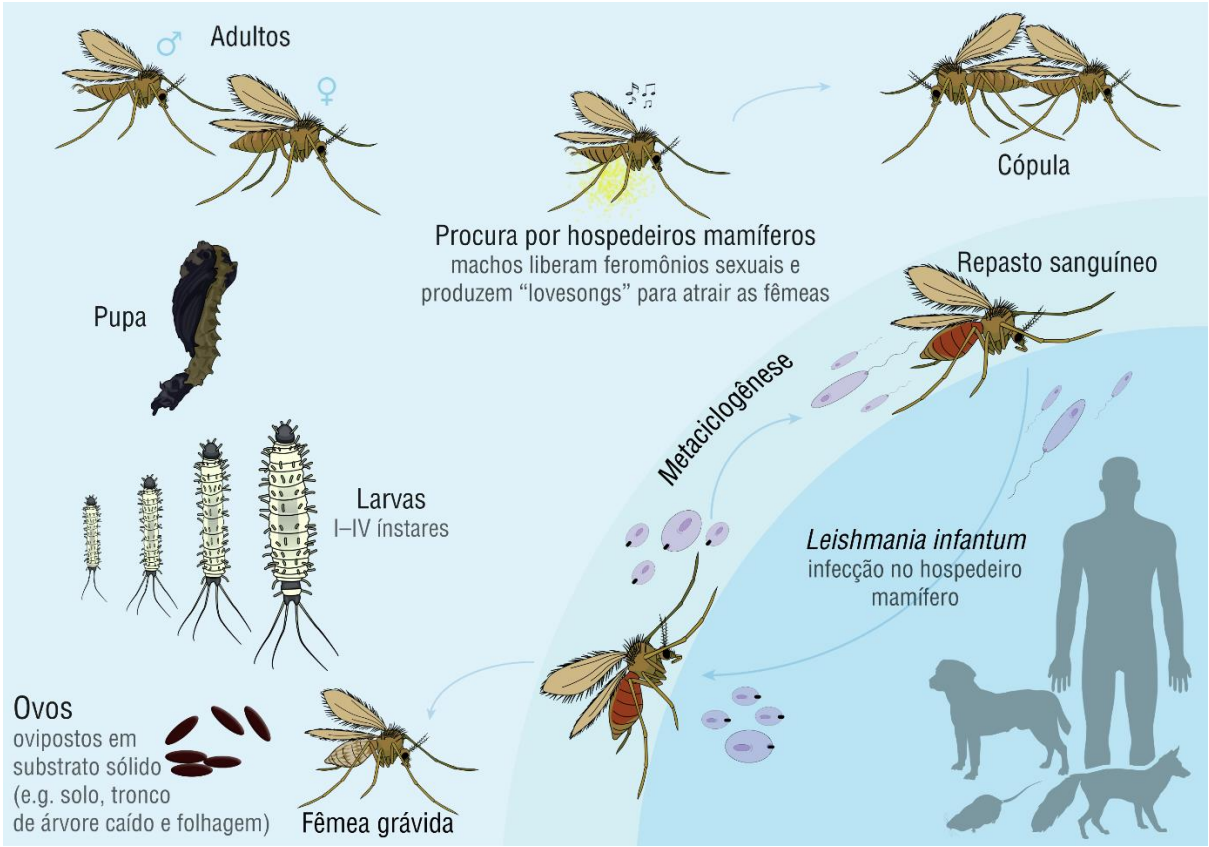
Os flebotomíneos — também conhecidos como flebótomos — são pequenos insetos pertencentes a ordem Diptera, família Psychodidae e subfamília Phlebotominae. Atualmente são aceitas aproximadamente 1.047 espécies de flebotomíneos no mundo, das quais 549 são encontradas no continente americano (GALATI, 2021; SHIMABUKURO; DE ANDRADE; GALATI, 2017). O Brasil, país com dimensões continentais, possui uma fauna flebotomínica rica e diversa com cerca de 292 espécies em seu território, aproximadamente um terço da fauna conhecida no mundo (GALATI, 2021).

Os flebotomíneos, apesar do seu tamanho, possuem grande relevância médico-veterinária (DANTAS-TORRES *et al.*, 2010). Com a capacidade de transmitir diversos patógenos — tais como bactérias, arbovírus e protozoários —, esses dípteros se destacam, principalmente, pelo seu importante papel no ciclo de transmissão das leishmânias (READY, 2013). Estima-se que algo em torno de 100 espécies atuam como vetores de *Leishmania* spp. em ambientes silvestres e domésticos (KILLICK-KENDRICK, 1999; RANGEL; LAINSON, 2009; MAROLI *et al*., 2013). Dentre essas, destaca-se *Lutzomyia longipalpis* (Lutz & Neiva, 1912), unanimemente apontado como o principal vetor de *Leishmania infantum* nas Américas (LAINSON; RANGEL, 2005; SALOMÓN *et al.*, 2015).

2.1 Bionomia de flebotomíneos neotropicais

Flebotomíneos são holometábolos, ou seja, apresentam um desenvolvimento completo de ovo, larvas, pupa e adultos (Figura 1). Além disso, todos os estágios de desenvolvimento dos flebotomíneos são estritamente terrestres, o que, por exemplo, os difere de outros dípteros hematófagos, tais como os mosquitos (LAWYER *et al.*, 2017).

Figura 1 — Ciclo de vida de *Lutzomyia longipalpis*.

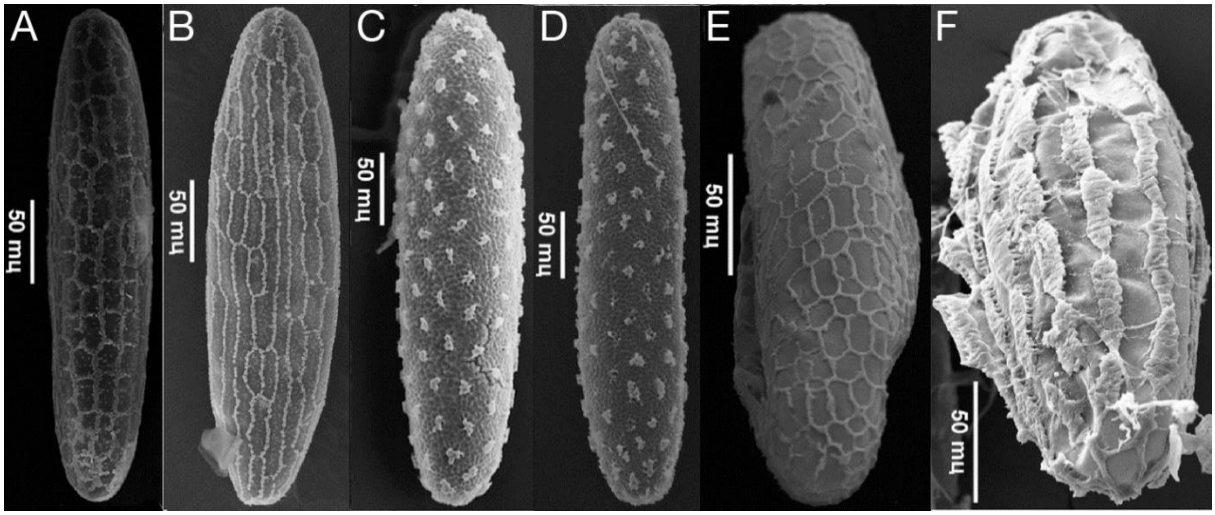


Fonte: Adaptado de Sousa-Paula, Otranto e Dantas-Torres (2020).

2.1.1 Formas imaturas: ovos, larvas e pupas

Os ovos dos flebotomíneos são oval-alongados, medindo entre 0,3–0,5 mm de comprimento e 0,1–0,15 mm de largura (Figura 2). A coloração varia de um branco-amarelado — logo após a oviposição — a marrom-escuro após algumas horas em contato com o ambiente. O exocório possui ornamentações que podem variar de acordo com a espécie, grupo e/ou gênero (PESSOA *et al.*, 2008). Estudos têm demostrado que, apesar de diferenças entre espécies, somente as ornamentações dos ovos não são suficientes para taxonomia de flebotomíneos (ALENCAR; SCARPASSA, 2018), apesar de gêneros e espécies morfologicamente próximas poderem ser distinguidos em alguns casos (ALMEIDA *et al.*, 2004; COSTA *et al.*, 2012).

Figura 2 — Microscopia eletrônica de varredura de ovos de flebotomíneos.

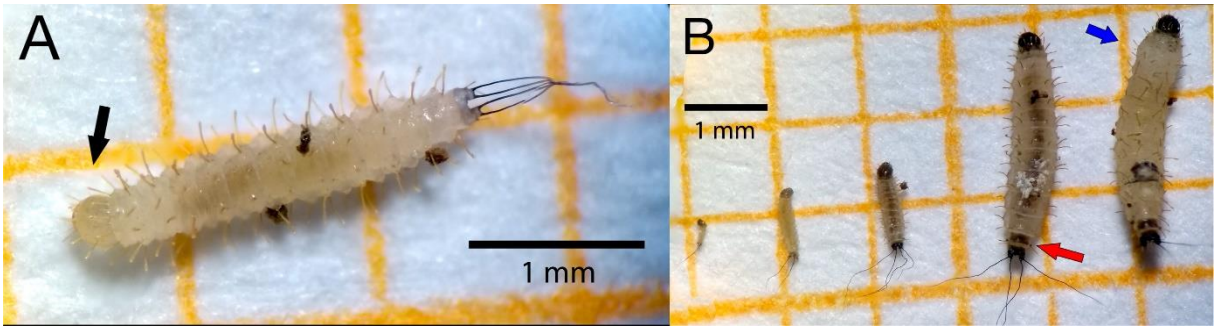


Fonte: Adaptado de Alencar e Scarpassa (2018).
Nota: Microfotografias mostrando as diferenças na ornamentação do exocório dos ovos das espécies (A) *Nyssomyia antunesi*, (B) *Nyssomyia whitmani*, (C) *Bichromomyia olmeca nociva*, (D) *Bichromomyia flaviscutellata*, (E) *Evandromyia walkeri* e (F) *Deanemyia samueli*.

Os ovos podem ser ovipostos juntos, ou separados, não havendo um padrão conhecido para que isso ocorra. Em média, uma fêmea produz cerca de 40 ovos por ciclo gonotrófico (BRAZIL; BRAZIL, 2018), podendo esse número variar amplamente. Estima-se que, na natureza, uma fêmea possa completar aproximadamente quatro ciclos gonotróficos (SERAFIM *et al*., 2021). A quantidade de ovos produzidos pode ser influenciada pela espécie, fonte de repasto sanguíneo e dieta das larvas (VOLF; VOLFOVA, 2011). Como mencionado anteriormente, o desenvolvimento de todos os estágios imaturos é estritamente terrestre. Sítios de oviposição podem variar amplamente, sendo principalmente micro habitats ricos em umidade, como abrigos de animais, frestas em troncos e rochas, solo contendo matéria orgânica em decomposição, e folhas (DEANE; DEANE, 1957; ALENCAR; FELICIANGELI, 2004; DE QUEIROZ; BARRETT, 2011; VIVERO *et al.*, 2015). Pela variedade de ambientes, difícil localização e visualização dos espécimes, o estudo de formas imaturas de flebotomíneos em seu habitat é fragmentado e pouco explorado (KILLICK-KENDRICK, 1999). Logo, isso implica na adoção de estratégias de controle, as quais na sua grande maioria são dirigidas às formas adultas, o que dificulta o controle de flebotomíneos e ações que reduzam o contato entre flebotomíneos, seres humanos e animais domésticos (SALOMÓN *et al.*, 2015; SOUSA-PAULA; OTRANTO; DANTAS-TORRES, 2020).

Geralmente entre seis e 10 dias após a oviposição as primeiras larvas eclodem. Larvas de flebotomíneos possuem o corpo alongado, com coloração branco-amarelado, com cabeça marrom-escuro — exceto logo após eclosão ou ecdise (Figura 3). O período larval é compreendido por quatro estádios ou instares, com o tamanho variado e outras características distintas (Figura 3). O primeiro estádio é extremamente pequeno, sendo de difícil visualização a olho nu. Larvas de primeiro estádio apresentam apenas duas cerdas caudais proeminentes. No segundo estádio, as larvas são maiores do que as do primeiro, e apresentam quatro cerdas caudais. Larvas de segundo e terceiro estádios podem ser facilmente confundidas, pois geralmente possuem tamanhos aproximados. Larvas de quarto estádio medem em torno de 4 mm e exibem uma característica placa anal dorsal esclerotizada (LAWYER *et al.*, 2017) (Figura 3b). Larvas de flebotomíneo possuem um aparelho bucal mordedor adaptado para uma alimentação baseada em matéria orgânica contida no solo (BRAZIL; BRAZIL, 2018). Próximo à muda para a fase de pupa, as larvas de quarto estádio param de se alimentar, e o abdômen esbranquiçado, indica a ausência de alimento no intestino (Figura 3b).

Figura 3 — Larvas de *Lutzomyia longipalpis*.

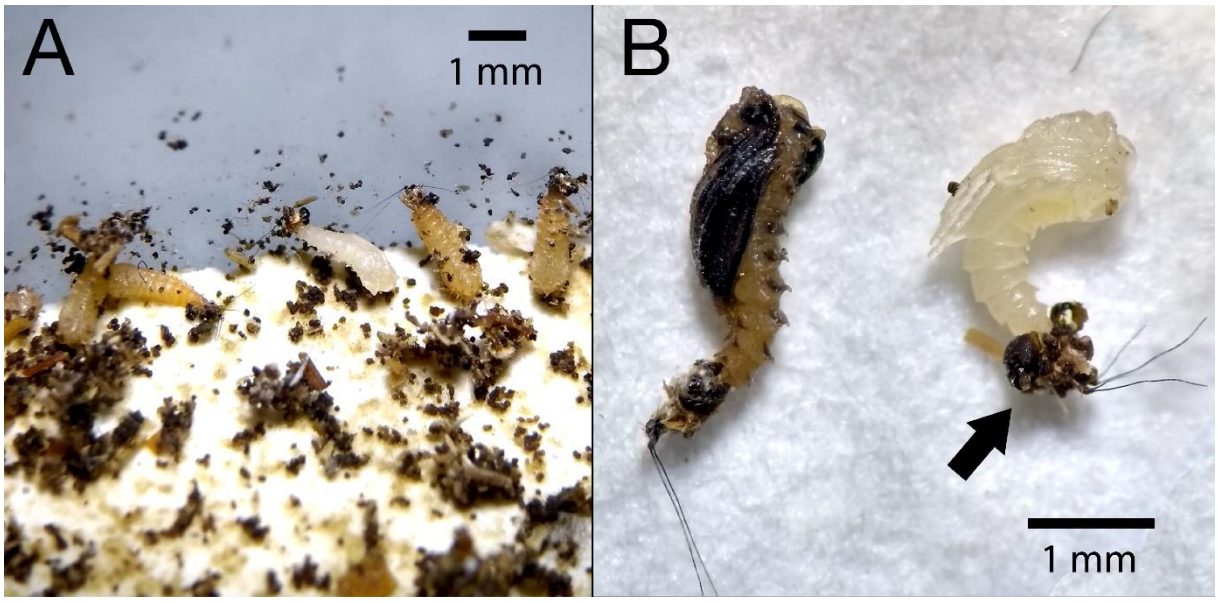


Fonte: O autor.
Nota: (A) Larva (3º estádio) após ecdise. Seta preta: cabeça com coloração esbranquiçada. (B) Larvas de 1º estádio (a esquerda), 2º, 3º e 4º estádio (a direita). Seta vermelha: placa anal dorsal esclerotizada. Seta azul: larva de 4º estádio próximo à muda para a fase de pupa.

As pupas de flebotomíneos possuem corpo semelhante às crisálidas de borboletas. A coloração varia de branco-amarelado no início do empupamento a amarelo ou avermelhado, ficando escuras próximo aos adultos emergirem (BRAZIL; BRAZIL, 2018) (Figura 4). O período pupal dura em média de seis a 10 dias, sendo a coloração uma característica que distingue a maturidade das pupas (VOLF; VOLFOVA, 2011). As pupas não se movem, permanecendo fixadas ao substrato.

Eventualmente, podem fazer rápidos movimentos de flexão e extensão (BRAZIL; BRAZIL, 2018). As pupas retêm a exúvia da larva, a qual cobre a sua porção caudal (Figura 4b). Apesar de não haver nenhum estudo explorando a função disso em flebotomíneos, em algumas espécies de maruins (Diptera: Ceratopogonidae) com desenvolvimento terrestre e morfologia das pupas semelhantes, essa característica está associada a proteção contra desidratação em habitats secos (SZADZIEWSKI; GIŁKA; ANTHON, 1995).

Figura 4 — Pupas de *Lutzomyia longipalpis*.



Fonte: O autor.
Nota: (A) Pupas de diferentes idades afixadas ao substrato do pote de criação. (B) Pupa madura, próximo o adulto emergir (a esquerda). A direita, pupa recente. Seta preta: exúvia da larva de 4º estádio.

2.1.2 Adultos

Flebotomíneos adultos medem em torno de 3 mm de comprimento, possuem corpo densamente coberto por cerdas, pernas longas e finas, e apresentam característicos voos curtos e saltitantes, bem como a manutenção das asas eretas — em formato de “V” — mesmo quando em repouso (KILLICK-KENDRICK, 1999).

Machos e fêmeas apresentam dimorfismo sexual, com diferenças tanto morfológicas, quanto comportamentais (BRAZIL; BRAZIL, 2018). A sexagem pode ser realizada, principalmente, através dos últimos segmentos abdominais (Figura 5). Apesar de diferenças morfológicas na cabeça (*e.g.,* partes bucais mais curtas nos machos e presença do cibário nas fêmeas) serem os principais caracteres que

também distinguem machos e fêmeas (BRAZIL; BRAZIL, 2018). Nas primeiras horas após muda imaginal, os machos possuem genitália rotacionada junta ao corpo, aproximadamente 24 horas depois, a genitália rotaciona-se 180°, indicando maturidade sexual (BRAZIL; BRAZIL, 2018). Todavia, é comum em condições de laboratório machos copularem com menos de 24 h de idade (LCS-P, observação pessoal). Do ponto de vista de monitoramento em habitat natural, a captura de machos com a genitália não-rotacionada pode indicar a proximidade da área de coleta a sítios naturais de criação de flebotomíneos (CARVALHO *et al.*, 2018).

Figura 5 — Fêmea e macho de *Lutzomyia longipalpis*

Fonte: O autor.
Nota: fêmea (à esquerda), macho (à direta).

Ambos machos e fêmeas precisam de uma dieta baseada em carboidratos para obtenção de energia (LIMA et al., 2016; FERREIRA et al., 2018). Os machos não possuem aparelho bucal adaptado para hematofagia, embora haja relatos pontuais de

machos ingurgitados com sangue em condições de laboratório (COELHO; FALCÃO; FALCÃO, 1966; GONTIJO *et al.*, 1987). Somente as fêmeas realizam repasto sanguíneo, o qual é necessário para a maturação dos ovos (BRAZIL; BRAZIL, 2018). Em algumas espécies, entretanto, são relatados processos de autogenia (fêmeas não precisam de sangue para produção de ovos) e partenogênese, ou seja, geração de prole sem inseminação do macho (KILLICK-KENDRICK, 1999; ALVES *et al.*, 2011), com pelo menos um relato de espécie com comportamento de telitoquia, ou seja, o ciclo de vida dessa espécie ocorre em total ausência de machos (BRAZIL; OLIVEIRA, 1999).

2.1.3 Reprodução

Os flebotomíneos apresentam um comportamento sexual complexo, o qual envolve estímulos e interações bioquímicas, acústicas e comportamentais (SOUSA-PAULA; OTRANTO; DANTAS-TORRES, 2020). Para algumas espécies (*e.g., L. longipalpis* s.l.), é observado um comportamento reprodutivo de *lekking* (KILLICK-KENDRICK, 1999). Os machos formam aglomerados e chegam primeiro ao hospedeiro vertebrado (KELLY; DYE, 1997). Então, liberam feromônios sexuais de agregação para, juntamente com o odor do hospedeiro, atrair as fêmeas para acasalamento — e, consequentemente, repasto sanguíneo —, o qual ocorre próximo ou sobre o hospedeiro (MORTON; WARD, 1989). Esses feromônios têm sido utilizados em diversas abordagens, em especial em estudos de complexos de espécies (vide tópico 2.4) e como novas estratégias para controle vetorial (COURTENAY *et al.*, 2019; GONZÁLEZ *et al.*, 2019). Durante o acasalamento, os machos batem suas asas com frequência, como comportamento de corte, agressividade contra outros machos e, em especial, produzindo sons durante a cópula (BRAY; HAMILTON, 2007). Além disso, esse comportamento é também por vezes associado a uma forma mecânica de dispersão de feromônios no ambiente (WARD *et al.*, 1988).

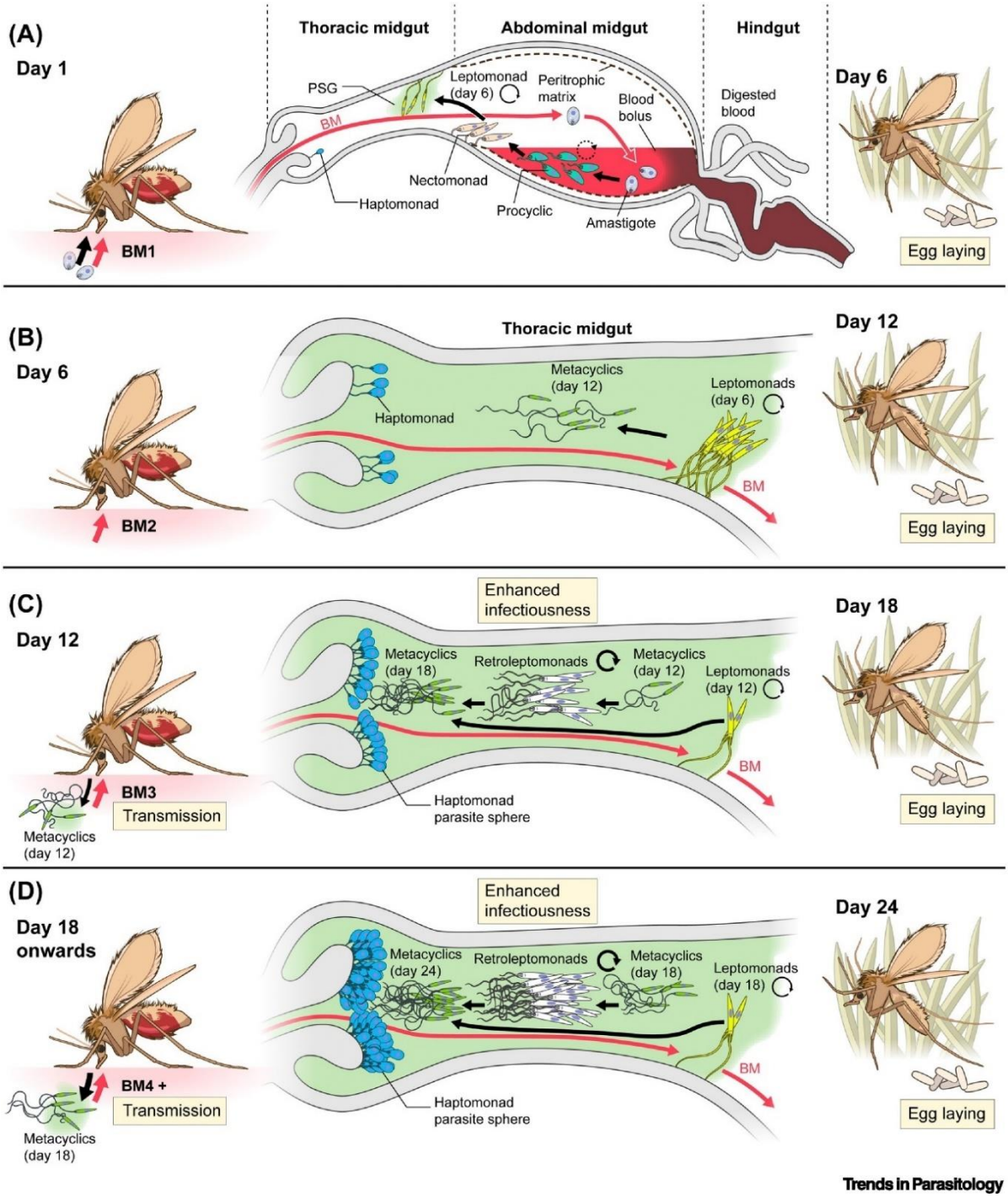
Inicialmente estudados em moscas-da-fruta (*Drosophila* spp.), sons copulatórios representam uma peça importante no reconhecimento sexual espécie-específico (GLEASON, 2005). Em *Drosophila* spp., membros de complexos de espécies produzem diferentes sons copulatórios, o que garante o isolamento reprodutivo entre grupos-irmãos (MARKOW; O'GRADY, 2005). Esses sons

copulatórios — juntamente com o aparato genético envolvido — vieram a ser uma ferramenta importante para estudos de complexos de espécies de flebotomíneos (OLIVEIRA *et al.*, 2001; PEIXOTO *et al.*, 2001; VIGODER *et al.*, 2013). Apesar de não possuírem um papel ainda totalmente compreendido, estudos sugerem que os sons copulatórios atuem no reconhecimento espécie-especifico de *L*. *longipalpis*, garantindo isolamento reprodutivo (pelo menos parcial) entre populações que produzem sons diferentes e, que, sejam determinantes no sucesso de inseminação das fêmeas (WARD *et al.*, 1983, 1988; SOUZA *et al.*, 2008; VIGODER *et al.*, 2020).

2.1.4 *Interação flebotomíneo*-Leishmania

Durante o repasto sanguíneo sobre um hospedeiro infectado, fêmeas de flebotomíneos podem ingerir formas amastigotas de *Leishmania* spp., as quais, uma vez no intestino do flebotomíneo, desenvolvem-se até sua forma infectante, a promastigota metacíclica, em um processo conhecido como metaciclogênese (KAMHAWI, 2006; BATES, 2018) (Figura 6).

Figura 6 — Ciclo natural da *Leishmania* spp. no flebotomíneo vetor.



Fonte: Adaptado de Serafim *et al*. (2021).

Nota: (A) fêmea de flebotomíneo realiza primeiro repasto sanguíneo (BM1) em um hospedeiro infectado com *Leishmania* spp., digere o sangue e realiza oviposição. No intestino abdominal, leishmânias diferenciam-se da forma amastigota para promastigota procíclica, seguindo para promastigota nectomona e, no dia 6 pós-repasto, promastigotas haptomona e leptomona, colonizando o intestino torácico e a válvula do estomodeu. (B) Fêmea realiza segundo repasto sanguíneo em um hospedeiro não-infectado. Leishmânias seguem se diferenciando e, ~12° dia pós-BM1, surgem as formas infectantes, promastigotas metacíclicas. (C) Fêmea realiza terceiro repasto sanguíneo (BM3) em um hospedeiro não-infectado, transmitindo leishmânias metacíclicas. O sangue ingerido promove a desdiferenciação de metacíclicas para a forma proliferativa promastigota retroleptomona, que em seguida diferenciam-se novamente em metacíclicas, aumentando consideravelmente a infecciosidade do flebotomíneo. (D) Fêmea de flebotomíneo realizada quarto repasto (BM4), dando continuidade no ciclo de transmissão das leishmânias.

Após o repasto sanguíneo, as formas amastigotas juntamente com o sangue ingurgitado são envolvidas pela matriz peritrófica (MP) dentro do tubo digestivo do flebotomíneo (PIMENTA *et al.*, 1997) (Figura 6-A). Posteriormente, amastigotas alongam seus corpos e expõem o flagelo, transformando-se em promastigotas procíclicas, as quais possuem rápida capacidade de multiplicação (WALTERS *et al.*, 1989). Antes do processo de digestão do bolo alimentar, formas procíclicas se transformam em nectomonas, que possuem flagelos mais longos e são mais móveis, o que possibilita o parasito se desvencilhar da MP e se afixar no epitélio intestinal (KAMHAWI, 2006). O êxito em aderir ao epitélio é um passo crucial para a sobrevivência dos parasitos e, consequentemente, está relacionado a competência de uma determinada espécie de flebotomíneo em transmitir determinada espécie de *Leishmania* (TEMPONE; PITALUGA; TRAUB-CSEKÖ, 2014).

Parte das leishmânias colonizam o intestino toráxico, assumindo outra forma proliferativa, as promastigotas leptomanas, e colonizam a válvula do estomodeu, promastigotas haptomonas (BATES, 2018). As haptomonas possuem flagelos mais curtos, pouca mobilidade e são responsáveis por formar um agregado de parasitos, conhecido como HPS (*haptomonads parasite sphere*), que obstrui a válvula do estomodeu. Após cerca de 12 dias, a metaciclogênese é concluída, com a presença da forma promastigota metacíclica (KAMHAWI, 2006). As leishmânias metacíclicas são o estágio infectante e possuem um longo flagelo, sendo bastante móveis e resistentes as defesa do hospedeiro vertebrado (HANDMAN; ELSO; FOOTE, 2005; KAMHAWI, 2006; WILSON *et al.*, 2010) (Figura 6-B). Durante um novo repasto sanguíneo realizado por uma fêmea de flebotomíneo infectada, formas metacíclicas são inoculadas no hospedeiro vertebrado (~12 dias pós-primeiro repasto), dando continuidade ao ciclo de transmissão das leishmânias (Figura 6-C). Recentemente foi descoberto que, ao realizar sucessivos repastos sanguíneos em um hospedeiro não-infectado, formas metacíclicas realizam um processo denominado como metaciclogênese reversa (SERAFIM *et al.*, 2018) (Figura 6-C). Nesse processo, parte dos parasitos metacíclicos se desdiferenciam em retroleptomonas, formas proliferativas que, após massiva proliferação, transformam-se novamente em promastigotas metacíclicas. Processo que aumenta exponencialmente a quantidade de formas metacíclicas no flebotomíneo, por sua vez potencializando a capacidade de transmissão (SERAFIM *et al.*, 2018, 2021) (Figura 6-D).

2.2 Métodos de incriminação de flebotomíneos vetores

A maioria das espécies de flebotomíneos possuem participação ainda desconhecida no ciclo de transmissão de *Leishmania* spp. Diversos fatores inerentes a biologia de cada espécie podem estar associados à sua competência em transmitir *Leishmania* spp. ao hospedeiro (KILLICK-KENDRICK, 1988, 1990; READY, 2013). No geral, estudos relacionados a bionomia da subfamília Phlebotominae são focados em alguns poucos grupos (MAROLI *et al.*, 2013). Consequentemente, investigações sobre a biologia da maioria das espécies são escassas, sobretudo em condições de laboratório, limitando nosso conhecimento sobre seus papéis como vetores (SOUSA-PAULA *et al.*, 2021).

Killick-Kendrick (1988) propôs quatro critérios para a incriminação de uma espécie de flebotomíneo como vetor de *Leishmania* spp.:

1 – Hábitos alimentares: o vetor deve se alimentar em hospedeiros reservatórios e, também, ser antropofílico, isto é, atraído por seres humanos;

2 – Infecção natural: uma forte evidência que a espécie possa ser um vetor, é o recorrente isolamento em fêmeas de flebotomíneos de campo da mesma *Leishmania* sp. isolada de pacientes.

3 – Distribuição: a espécie de flebotomíneo tem uma distribuição sobreposta aos casos humanos e reservatórios.

4 – Competência vetorial: a espécie de flebotomíneo suporta o desenvolvimento da *Leishmania* sp. em questão e a transmite através da picada.

Esses critérios foram revisados por Ready (2013), o qual sugeriu também o uso de modelagens matemáticas para demonstrar se a espécie de flebotomíneo é essencial para a manutenção da doença na área e se a incidência da doença diminui à medida que a taxa de picada pelo vetor putativo também reduz. Apesar dos critérios supracitados serem amplamente aceitos, pode haver certa subjetividade, eventualmente causando dubiedade. Por exemplo, em condições de laboratório a espécie do Velho Mundo *Leishmania major* se desenvolve em *L*. *longipalpis*, o qual não é um vetor natural do parasito (MYSKOVA *et al.*, 2007). Portanto, pode haver confusão em relação ao critério 2 de Killick-Kendrick (1988), no qual a espécie de flebotomíneo tem que ser infectada naturalmente com a mesma *Leishmania* sp. isolada dos pacientes. Em vista disso, Maroli et al. (2013) propõem que para uma

incriminação robusta, o vetor putativo deve apresentar: (i) evidências epidemiológicas comprovando uma sazonalidade sobreposta entre o vetor e a ocorrência da doença em humanos; (ii) comportamento antropofílico; e (iii) evidências de que haja in natura o desenvolvimento de *Leishmania* sp. no intestino da espécie de flebotomíneo suspeita. Somado a isso, em áreas endêmicas para transmissão da doença, as evidências podem ser reforçadas quando há ausência de um vetor conhecido.

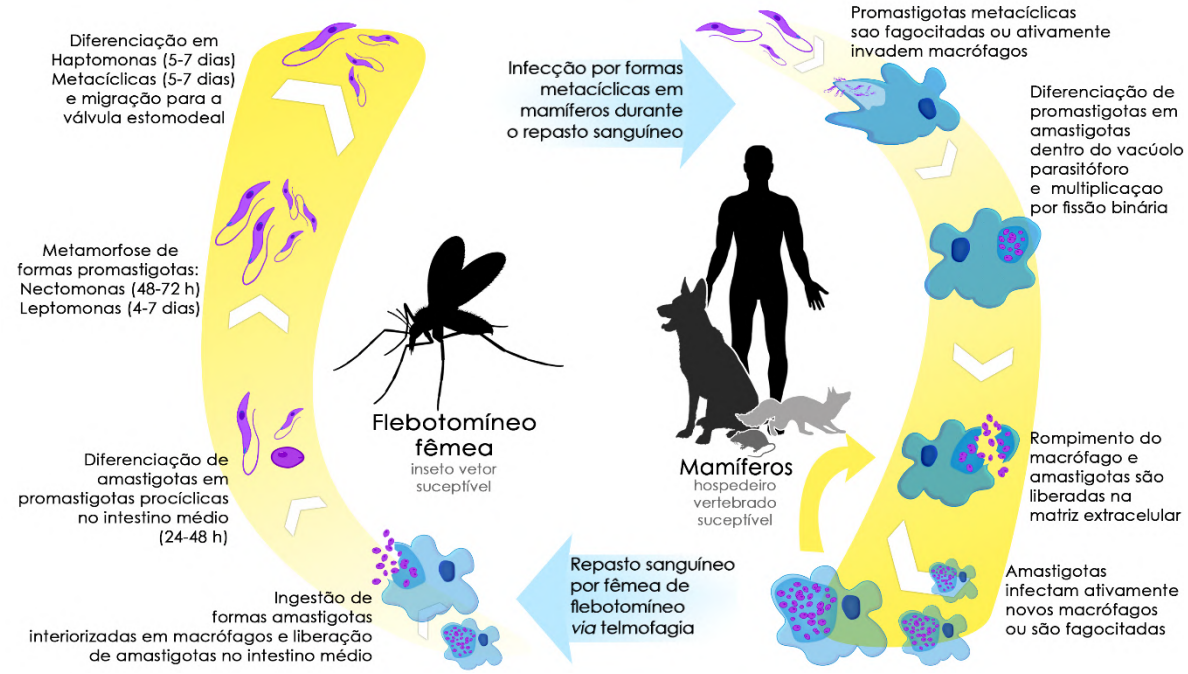
2.3 *Leishmania* spp. e as leishmanioses

As leishmanioses representam um importante problema de saúde pública com mais de um bilhão de pessoas vivendo em áreas de risco para infecção (WORLD HEALHT ORGANIZATION, 2017). Elas compreendem um complexo grupo de doenças que manifestam distintas formas clínicas (KAMHAWI, 2006). Existem três formas principais: a leishmaniose tegumentar (LT), a forma mais comum; leishmaniose visceral (LV), a forma mais severa e potencialmente letal quando não tratada; e a leishmaniose mucocutânea, a forma mais incapacitante e estigmatizante (ALVAR *et al.*, 2012).

As leishmanioses são causadas por protozoários do gênero *Leishmania* (Ross, 1903), pertencentes à família Trypanosomatidae, sendo todos os membros dessa família associados à artrópodes, mamíferos e/ou outros vertebrados (BURZA; CROFT; BOELAERT, 2018) (

Figura 7).

Figura 7 — Ciclo de vida digenético de *Leishmania* spp.



Fonte: O Autor.

Atualmente mais de 50 espécies de leishmânias (*Leishmania* spp.) são descritas em todo o mundo (ESPINOSA *et al.*, 2018). O gênero *Leishmania* é subdivido em quatro subgêneros: *Leishmania*; *Viannia*; *Sauroleishmania*; e *Mundinia* (ESPINOSA *et al.*, 2018). Os subgêneros *Leishmania* e *Viannia* reúnem os principais causadores de zoonoses, diferindo entre si, principalmente, pela região onde se multiplicam no trato digestivo do inseto vetor (LAINSON; SHAW, 1987). Em todo o mundo, aproximadamente 23 espécies de *Leishmania* são humano-patogênicas, dentre as quais *L. infantum*, causadora da LV no continente americano (ESPINOSA *et al.*, 2018).

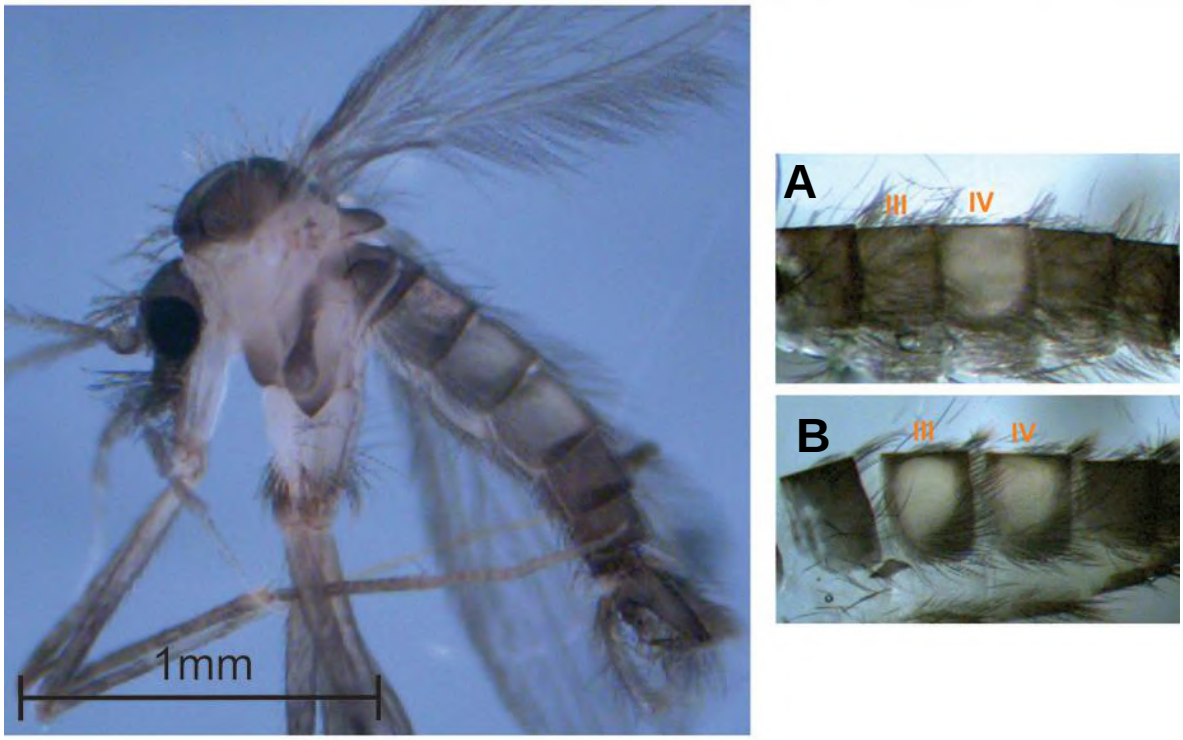
A dispersão das espécies de *Leishmania* é intimamente associada à distribuição geográfica de seus flebotomíneos vetores. Ambos subgêneros, *Leishmania* e *Viannia*, são reportados e transmitidos nas Américas por fêmeas de flebotomíneos dos gêneros *Bichromomyia*, *Lutzomyia*, *Migonemyia*, *Nyssomyia* e *Psycodopygus* (DANTAS-TORRES *et al.*, 2012; RANGEL *et al.*, 2018; SOUSA-PAULA *et al.*, 2021).

2.4 O complexo *Lutzomyia longipalpis*

Lutzomyia longipalpis foi descrita em 1912 por Adolpho Lutz e Arthur Neiva como *Phlebotomus longipalpis*, com base em um único espécime macho e algumas (número incerto) fêmeas coletadas nos estados de Minas Gerais e São Paulo (LUTZ; NEIVA, 1912). Quase uma década depois, França (1920) criou o gênero *Lutzia* para a espécie, substituindo o nome posteriormente por *Lutzomyia* — *Lutzia* já era um nome pré-ocupado para um gênero de mosquito, *Lutzia* Theobald (1903) (FRANÇA, 1924). O táxon *Lutzomyia longipalpis* não foi alterado desde então (SOUSA-PAULA; DANTAS-TORRES, 2021).

Em observações pioneiras, Mangabeira Filho (1969) relatou variações fenotípicas entre machos de *L*. *longipalpis* coletados em dois estados brasileiros, Pará e Ceará. Os espécimes do Pará exibiam um único par de manchas pálidas dorsolaterais no IV tergito abdominal, enquanto os do Ceará possuíam dois pares no III e IV tergitos abdominais. Posteriormente, convencionou-se chamar tais características como fenótipos 1S e 2S (do inglês *one spot* e *two spots*, respectivamente) (Figura 8) (WARD *et al.*, 1988). Apesar de não ter ficado claro para Mangabeira Filho se tais diferenças representavam variações da espécie (intraespecífica) ou de duas espécies distintas (interespecífica), posteriormente essa veio a ser a primeira evidência de que poderiam existir diferentes táxons sob a espécie nominal *L*. *longipalpis*.

Figura 8 — Fenótipos 1S e 2S dos machos de *Lutzomyia longipalpis* sensu lato.



Fonte: Adaptado de Souza, Brazil, Araki (2017).
Nota: Vista lateral. exibindo (A) fenótipo 1S e (B) fenótipo 2S. III e IV indicam a denominação dos tergitos abdominais.

Na década de 1970, colônias de *L*. *longipalpis* com espécimes capturados na caverna da Lapinha, Minas Gerais (fenótipo 1S) e Morada Nova, Ceará (fenótipo 2S) foram estabelecidas na Inglaterra (KILLICK-KENDRICK; LEANEY; READY, 1973; WHITE; KILLICK-KENDRICK, 1975). Ao realizar cruzamentos entre as duas populações alopátricas, observou-se um certo nível de infertilidade, com baixa taxa de larvas eclodindo. Inicialmente, análises de cromossomos politênicos indicaram nenhuma diferença entre as duas populações (WHITE; KILLICK-KENDRICK, 1975). Posteriormente, Ward *et al*. (1983) repetiram os cruzamentos entre as populações de Lapinha (1S) e Morada Nova (2S). Adicionalmente, realizaram cruzamentos com mais duas populações simpátricas coletadas em Sobral, Ceará (Sobral 1S x Sobral 2S) e entre uma população de Ilha de Marajó, Pará (1S) com Lapinha (1S). Os resultados mostraram um isolamento reprodutivo parcial entre as populações, com pelo menos duas formas sexualmente distintas. A evidência de barreiras zigóticas — sobretudo entre populações simpátricas —, adicionou peso à hipótese que *L*. *longipalpis* poderia constituir um complexo de espécies crípticas. Além disso, não fugiu aos olhos dos

autores que membros do complexo poderiam ter papéis vetoriais distintos na transmissão de *L*. *infantum* (WARD *et al.*, 1983).

Uma série de estudos foi realizada a partir de então. Lane e Ward (1984) investigaram a possível função das manchas nos tergitos de ambos os fenótipos 1S e 2S de *L*. *longipalpis*. Análises de microscopia eletrônica indicaram que na região abdominal das manchas poderia existir glândulas secretoras de feromônios. A função secretora das glândulas fora posteriormente confirmada, além de se tratar de feromônios sexuais (LANE, R. *et al.*, 1985; PHILLIPS *et al.*, 1986; WARD *et al.*, 1989). Além disso, esses feromônios — liberados antes e durante a cópula com fêmeas — poderiam ser dispersados mecanicamente no ambiente pela frenética batida das asas dos machos durante a cópula com as fêmeas (MORTON; WARD, 1989; NIGAM; WARD, 1991). A partir de então, o estudo dos feromônios sexuais se tornou particularmente importante para o estudo do complexo *L*. *longipalpis* (SPIEGEL *et al.*, 2016). Por outro lado, Phillips *et al*. (1986), analisando os componentes químicos dos feromônios de quatro populações brasileiras (Sobral-CE 1S e 2S; Ilha de Marajó-PA 1S; Santarém-PA1S), encontraram dois tipos distintos de componentes; por sua vez, os tipos de feromônios não estavam correlatos aos fenótipos das manchas apresentados pelos machos. Os autores sugeriram, portanto, que somente a identificação do número das manchas nos tergitos abdominais não poderia ser um marcador suficiente para distinguir entre populações reprodutivamente isoladas de *L. longipalpis*.

A partir da década de 1990, análises mais robustas, considerando um número maior de populações, foram realizadas para elucidar os tipos de feromônios sexuais produzidos e suas distribuições entre populações de *L. longipalpis* s.l. (HAMILTON; WARD, 1991; HAMILTON; DOUGHERTY; WARD, 1994; HAMILTON *et al.*, 1999; PALFRAMAN *et al.*, 2018). No total, pelo menos cinco tipos diferentes de feromônios são produzidos por machos do complexo. Apesar de não haver uma distribuição geográfica clara para cada tipo, populações com o feromônio (*S*)-9-methyl-germacrene-B (9MGB) se concentram principalmente na América do Sul e Central, seguido por cembrene-1, cembrene-2 e (*1S*,*3S*,*7R*)-3-methyl-α-himachalene (3MαH) (SPIEGEL *et al.*, 2016). Sobralene, o mais recente descoberto, somente foi analisado em populações de Sobral-CE 2S (PALFRAMAN *et al.*, 2018).

Com as evidências que populações de *L*. *longipalpis* s.l. poderiam ter um fluxo gênico interrompido — mediado por feromônios e comportamentos sexuais —,

análises de estruturação genética começaram a investigar se tais populações poderiam representar também grupos divergentes. Lanzaro *et al*. (1993) combinaram análises de esterilidade dos machos híbridos com análises de isoenzimas de populações do Brasil (Lapinha, MG), Colômbia e Costa Rica. Os resultados das diferenças genéticas foram consideravelmente altos entre os três grupos e a progênie de machos híbridos apresentou espermatozoides anormais. Posteriormente, outros estudos mostraram que as mesmas populações diferiam também na quantidade produzida do vasodilatador *maxadilan* (populações do Brasil e Colômbia possuíam 10–40 vezes mais *maxadilan* do que a população da Costa Rica), na estrutura de aminoácidos do peptídeo, e também na quantidade de RNAm transcrito (LANZARO, G. C. *et al.*, 1999; WARBURG *et al.*, 1994; YIN; NORRIS; LANZARO, 2000). Esses dados estavam de acordo com manifestações clínicas incomuns para infecções por *L. infantum* encontradas na Costa Rica: locais onde a espécie *L*. *longipalpis* era predominante, infecções com *L. infantum* causavam uma atípica leishmaniose cutânea, e não lesões viscerais. Assim, sugeriu-se que populações do complexo *L*. *longipalpis* poderiam, também, atuar de forma distinta na imunopatogenicidade das leishmanioses.

O uso de marcadores genéticos para estudos com o complexo cresceu ao longo dos anos, o que contribuiu consideravelmente para um melhor entendimento da sua variação genética e padrões de evolução. Embora vários estudos reforçassem a existência de espécies crípticas sob o táxon *L. longipalpis* na região Neotropical (DUJARDIN *et al.*, 1997; LANZARO *et al.*, 1998; SOTO *et al.*, 2001), alguns estudos mostravam dados contraditórios, apontando para a existência de uma única espécie, porém altamente polimórfica (MUKHOPADHYAY *et al.*, 1998a, 1998b; MUTEBI *et al.*, 1998; RANGEL *et al.*, 1999; DE QUEIROZ BALBINO *et al.*, 2006; AZEVEDO *et al.*, 2008).

Dujardin *et al*. (1997) observaram que, além da genética, haviam variações morfométricas nas asas de populações com fenótipos 1S e 2S da Bolívia, e que isso poderia ter significância taxonômica. Outros estudos indicaram diferenças morfométricas em partes bucais de larvas de populações venezuelanas (ARRIVILLAGA, *et al.*, 2000; ARRIVILLAGA; FELICIANGELI, 2000; ARRIVILLAGA; RANGEL; FELICIANGELI, 2000). Posteriormente, Arrivillaga e Feliciangeli (2001) propuseram a descrição de *Lutzomyia pseudolongipalpis* Arrivillaga & Feliciangeli, 2001 com base em caracteres morfológicos de fêmeas adultas. Apesar dos machos

serem morfologicamente indistinguíveis, diferenças nos dentes do cibário, asas e genitália das fêmeas distinguem a população de La Rinconada (Venezuela) de fêmeas de *L*. *longipalpis* tanto da Venezuela, quanto de outras regiões.

A partir da década de 2000, estudos focados no comportamento e genética dos sons copulatórios produzidos por machos do complexo começaram a ser desenvolvidos (SOUZA, Nataly A; BRAZIL; ARAKI, 2017). Em *Drosophila melanogaster* (Diptera: Drosophilidae), pulsos e intervalos entre pulsos gerados pelos sons das asas são controlados pelos genes *period* (*per*) e *cacophony* (*cac*) (GLEASON, 2005). Esses ritmos fazem parte do reconhecimento sexual espécie-específico, estando, portanto, relacionados ao isolamento reprodutivo entre espécies filogeneticamente e morfologicamente muito próximas (grupos-irmãos) (OLIVEIRA *et al.*, 2001).

Estudos identificaram variações genéticas nos genes *period* e cacophony entre populações alopátricas de Lapinha, Jacobina e Natal de *L*. *longipalpis*, bem como entre populações simpátricas de 1S e 2S de Sobral (OLIVEIRA et al., 2001; BAUZER et al., 2002a, 2002b; BOTTECCHIA et al., 2004). Análises dos sons produzidos revelaram que esses eram muito distintos entre algumas populações (SOUZA, *et al.*, 2002; SOUZA, *et al.*, 2004). Pelo menos três tipos de sons são produzidos pelos machos: *Pulse*, *Burst* e *Mix* (VIGODER *et al.*, 2013). O agrupamento de populações de acordo com o tipo de som produzido são congruentes com as variações genéticas encontradas nos genes *period* e *cacophony* (SOUZA *et al.*, 2002; SOUZA *et al.*, 2004). Além disso, foi observado também isolamento reprodutivo tanto entre populações alopátricas, quanto simpátricas (SOUZA *et al.*, 2008).

Análises de polimorfismos fixados no gene *period* indicaram que populações simpátricas de 1S e 2S de três estados do nordeste brasileiro (Sobral-CE; Bodocó-PE; e Caririaçu-PI) possuem uma estruturação genética entre os fenótipos 1S e 2S (LIMA COSTA *et al.*, 2015). Aparentemente, quando ocorrem em simpatria, os fenótipos das manchas nos machos podem auxiliar na identificação entre os membros do complexo, mas sendo imprescindível o uso de outros marcadores (*e.g.,* genéticos). Além disso, os mesmos polimorfismos fixados encontrados nos machos também podem ser utilizados para identificação das fêmeas correspondentes aos machos (DE SOUZA FREITAS *et al.*, 2018).

Um dos estudos mais conclusivos sobre a existência de espécies crípticas em *L*. *longipalpis* utilizou uma abordagem integrada de marcadores moleculares, sons de

corte e feromônios (ARAKI *et al.*, 2009, 2013). Os dados mostraram que, pelo menos entre populações brasileiras, há pelo menos dois principais grupos: um mais homogêneo, formado por populações que produzem o som de cópula *Burst* e feromônio cembrene-1; enquanto um segundo grupo é mais heterogêneo, produzindo cinco variações do som *Pulse* e três tipos de feromônios. O número de espécies crípticas no segundo grupo ainda é incerto (ARAKI *et al.*, 2013). Atualmente, é reconhecido que a espécie nominal *L*. *longipalpis* abriga um número incerto de espécies crípticas que coletivamente são referidas como *L*. *longipalpis* sensu lato (BAUZER *et al.*, 2007; SOUZA; BRAZIL; ARAKI, 2017; SOUSA-PAULA *et al.*, 2021), com provavelmente várias espécies no Brasil (VIGODER *et al.*, 2015). Todavia, nenhuma das espécies brasileiras foram devidamente descritas e receberam nomes válidos até então (BRANDÃO-FILHO *et al.*, 2009; SOUSA-PAULA; DANTAS-TORRES, 2021). Além de *L*. *longipalpis* s.l., outras espécies de flebotomíneos neotropicais compõem o referido complexo *L*. *longipalpis*, as quais podem desempenhar papéis distintos e/ou desconhecidos no ciclo de transmissão de espécies de leishmânias (SOUSA-PAULA *et al.*, 2021).

As abordagens utilizando sons copulatórios e polimorfismos no gene *period* ajudaram no reconhecimento de *Lutzomyia cruzi* Mangabeira, 1938 como um outro membro do complexo *L*. *longipalpis* (VIGODER *et al.*, 2010). Fêmeas de *L*. *cruzi* e *L. longipalpis* são indistinguíveis, apesar de machos serem diferenciados através das cerdas que constituem o tufo do segmento basal do gonocoxito (MANGABEIRA FILHO, 1969). Além disso, fêmeas de *Lutzomyia gaminarai* Cordero, Vogelsang & Cossio, 1928 e *Lutzomyia matiasi* Le Pont & Mollinedo, 2009 também são idênticas às fêmeas de *L*. *longipalpis* (GALATI, 2018). Portanto, essas duas espécies devem ser consideradas membros do complexo (BAUZER *et al.*, 2007; SOUSA-PAULA *et al.*, 2021). Outra espécie que pertencem ao complexo *L*. *longipalpis* é *Lutzomyia alencari* Martins, Souza & Falcão, 1962 (SOUSA-PAULA *et al.*, 2021). Embora ambos machos e fêmeas de *L*. *alencari* e outros membros do complexo *L*. *longipalpis* sejam distinguíveis morfologicamente (GALATI, 2018), estudos genéticos apontam para uma proximidade filogenética entre *L*. *alencari* e *L*. *longipalpis* s.l. (PINTO *et al.*, 2015).

Estudos motivados pelo interesse taxonômico do complexo *L*. *longipalpis* têm proporcionado grandes avanços no entendimento da biologia desse grupo, principalmente do ponto de vista evolutivo (BAUZER *et al.*, 2007; SPIEGEL *et al.*, 2016; SOUZA; BRAZIL; ARAKI, 2017). Nenhuma descrição formal das espécies

crípticas brasileiras foi feita até então, apesar de todos os estudos supracitados e discussões entre especialistas (BRANDÃO-FILHO *et al.*, 2009; SOUSA-PAULA; DANTAS-TORRES, 2021). Por outro lado, o reconhecimento de espécies críticas vai além de uma questão taxonômica, sobretudo entre vetores de doenças, tendo em vista como membros de complexos de espécie podem ter papéis vetoriais distintos (LANZARO *et al.*, 1999; MAINGON *et al.*, 2008; SOUSA-PAULA *et al.*, 2021). Até então, são escassos os estudos que investiguem se de fato diferentes membros do complexo *L*. *longipalpis* possuem papéis vetoriais distintos (LANZARO *et al*., 1999; YIN; NORRIS; LANZARO, 2000; MAINGON *et al*., 2008; CASANOVA *et al*., 2015). Mais estudos voltados para a eco-biologia do complexo *L*. *longipalpis* poderão revelar diferenças substanciais que, juntamente com os dados já existentes, auxiliarão no reconhecimento de espécies, o que pode influenciar diretamente nas medidas de controle e prevenção das leishmanioses.

3 OBJETIVO GERAL

Avaliar e comparar aspectos de bionomia, genética e compatibilidade reprodutiva de três populações de *Lutzomyia longipalpis* s.l.

3.1 Objetivos específicos

a. Avaliar a estruturação genética de populações de *L*. *longipalpis* s.l. através de análises empregando o gene *period* como marcador;
b. Avaliar a compatibilidade biológica das populações através de cruzamentos interespecíficos.
c. Comparar os ciclos de vida de populações de *L*. *longipalpis* s.l. sob condições de laboratório;

4 RESULTADOS

Nessa seção são apresentados os artigos publicados na íntegra que compõe os resultados referentes aos objetivos da tese.

4.1 Artigo 1 – Beyond taxonomy: species complexes in New World phlebotomine sand flies

Nesse artigo publicado em *Medical and Veterinary Entomoly*[1], nós destacamos os complexos de espécies em flebotomíneos do continente americano. Considerando mais de 540 espécies válidas de flebotomíneos no Novo Mundo, aproximadamente 30% apresentam machos, fêmeas ou ambos os sexos morfologicamente indistinguíveis de uma ou mais espécies. Essas espécies indistinguíveis formam complexos de espécies, *i.e.,* arranjos informais de espécies que são filogeneticamente próximas e, que, eventualmente são morfologicamente similares, quando não idênticas. Há de se notar que o papel como agente transmissor de patógenos dentro de um complexo de espécie pode variar entre seus membros. Nós discutimos aspectos pertinentes a identificação desses complexos e suas possíveis implicações dentro do ciclo de transmissão de patógenos.

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REVIEW ARTICLE

Beyond taxonomy: species complexes in New World phlebotomine sand flies

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Abstract. A species complex (= species group, species series) is an assemblage of species, which are related morphologically and phylogenetically. Recent research has revealed several arthropod vector species that were believed to be a single nominal species actually representing a group of closely related species, which are sometimes morphologically indistinguishable at one or more developmental stages. In some instances, differences in terms of vector competence, capacity, or both have been recorded. It highlights the importance of detecting and studying species complexes to improve our understanding of pathogen transmission patterns, which may be vectored more or less efficiently by different species within the complex. Considering more than 540 species, about one-third of the phlebotomine sand flies in the New World present males and/or females morphologically indistinguishable to one or more species. Remarkably, several of these species may act in transmission of pathogenic agents. In this article, we review recent research on species complexes in phlebotomine sand flies from the Americas. Possible practical implications of recently acquired knowledge and future research needs are also discussed.

Key words. Cryptic species, morphology, Phlebotominae, taxonomic problem, vector-borne disease, vector competence, vectors.

Introduction

Many arthropods have blood-based diets, sucking blood from a range of different hosts, including wild and domestic animals, as well as humans (Marcondes, 2017). When taking a blood-meal, some arthropods can eventually transmit pathogenic organisms to their hosts, such as bacteria, helminths, protozoa and viruses. According to World Health Organization, vector-borne diseases cause more than 700 000 deaths annually, representing 17% of all infectious diseases around the world (WHO, 2020). Notwithstanding, most of these diseases can be prevented by control and surveillance measures, which, in turn, involve the correct identification of the vector species.

Arthropoda is the most speciose phylum within the kingdom Animalia, representing around ~80% of the diversity of all known animals (Zhang, 2013). Arthropods occupy virtually every marine, freshwater, terrestrial and aerial habitat on Earth (Black IV & Kondratieff, 2005). Nonetheless, its actual biodiversity is underestimated considering that many groups are of minor economic or medical and veterinary significance, thus oftentimes neglected by taxonomists. Moreover, some arthropod groups show a high cryptic diversity and taxonomic problems (Brown, 1959; Trontelj & Fier, 2009; Poulin & Pérez-Ponce de León, 2017), which may have practical implications when species complexes refer to arthropod vectors of pathogens (Bickford *et al.*, 2007).

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We define species complex (also referred to as 'species groups' or 'species series') as an informal assemblage of taxa, whose members are related phylogenetically and share morphological similarities (i.e., they resemble each other). Eventually, such traits might be considered insufficient to create new formal categories (e.g., genus or subgenus) for grouping them. In some cases, members of a species complex may display few or no phenotypic differences, being virtually indistinguishable on morphological grounds at one or more developmental stages (e.g., Hebert *et al.*, 2004; Elmer *et al.*, 2010; Nava *et al.*, 2014; Galati, 2018). Species that are morphologically indistinguishable, but belong to distinct evolutionary lineages are referred to as cryptic species (Sáez & Lozano, 2005; Bickford *et al.*, 2007; Gill *et al.*, 2016; Struck *et al.*, 2018); thus, a cryptic species complex is a species complex that includes morphologically indistinguishable species. Because cryptic species are morphologically identical, they may remain undescribed for years (Bickford *et al.*, 2007).

Incidentally, the term cryptic species has been interchangeably referred as a synonym with other terms, such as sibling species (Struck *et al.*, 2018). According to Mayr (1963), sibling species are 'morphologically exceedingly similar, if not identical, but are reproductively isolated'. Paradoxically, studies on crossbreeding and hybridization may be scarce for some important disease vectors [e.g., phlebotomine sand flies (dos Reis & Alevi, 2020)]. In this regard, a species complex may include cryptic, sibling and morphologically similar species, but not necessarily all members of the complex must be cryptic- and/or sibling species.

Interestingly, the existence of species complexes is a fairly common phenomenon in arthropod vectors, and it has been reported within biting midges (Diptera: Ceratopogonidae) (Harrup *et al.*, 2015; Mignotte *et al.*, 2020), black flies (Diptera: Simuliidae) (Adler *et al.*, 2010), mosquitoes (Diptera: Culicidae) (Harbach, 2012; Harbach, 2013), phlebotomine sand flies (Diptera: Psychodidae) (Souza *et al.*, 2017; Galati, 2018; this present review), ticks (Ixodida: Ixodidae) (Nava *et al.*, 2014; Dantas-Torres, 2018; Guglielmone *et al.*, 2020), triatomine bugs (Hemiptera: Reduviidae) (Schofield & Galvão, 2009; Santillán-Guayasamín *et al.*, 2020) and tsetse flies (Diptera: Glossinidae) (Dorn *et al.*, 2017). In the same way, studies have demonstrated that members of these species complexes may display differences in terms of vector capacity or competence. For instance, the *Anopheles gambiae* Giles complex includes nine described species (Sinka *et al.*, 2010; Dorn *et al.*, 2017), of which *Anopheles gambiae* sensu stricto is the primary vector of *Plasmodium falciparum* Welch in the sub-Saharan Africa (Sinka *et al.*, 2010). This mosquito is notable for being endophilic and anthropophilic (Sinka *et al.*, 2010; Dorn *et al.*, 2017). In contrast, *Anopheles quadriannulatus* Theobald (another member of the *An. gambiae* complex) feeds preferentially on cattle rather than humans (Pates *et al.*, 2001; Besansky *et al.*, 2004), which greatly decreases its vector capacity to transmit *P. falciparum*. As such, it is suggested that *An. quadriannulatus* may play no role in transmission of this pathogen, even if it is a permissive vector under laboratory conditions (Takken *et al.*, 1999; Fontaine *et al.*, 2015; Habtewold *et al.*, 2017). From a medico-veterinary viewpoint, this fact highlights the importance for assessing and naming cryptic species within the phylum Arthropoda, since misidentifications of vector species may have eminent implications for animal and human health.

Herein, we review and discuss the recent studies on species complexes in vectors of medical and veterinary importance, with special reference to New World phlebotomine sand flies. We also highlight possible practical implications of recently acquired knowledge and pointing out to future research needs.

A complex species or a species complex?

'No one definition has satisfied all naturalists', wrote Darwin many years ago about the various species concepts in the classic *On the Origin of Species* (Darwin, 1859). And the debate concerning species concept and species delimitation endures (Wheeler & Meier, 2000; De Queiroz, 2005, 2007). Indeed, more than 30 alternative species concepts have been proposed (Zachos, 2016), with the biological, ecological, phylogenetic and typological species concepts being the most widely used (De Queiroz, 2007). Likewise, the subspecies rank is also an issue of debate (Patten, 2015). In general, subspecies are designated as subsets (or populations) morphologically similar of a given taxon which are geographically isolated (allopatric) and, eventually, are able to interbreed at zones of contact (parapatric) generating fertile offspring (Braby *et al.*, 2012). Nonetheless, subspecies may be considered as an arbitrary category (Wilson & Brown, 1953), being variations – morphotypes – of a nominal species which the available data are incongruent and/or inconclusive to elevate them to a specific rank (e.g., Depaquit *et al.*, 2013).

As a matter of fact, one of the grand challenges in zoological taxonomy in the post-genomic Era is still what defines a species. From a pragmatic standpoint, the International Code for Zoological Nomenclature provides a set of rules for the naming of animal species (ICZN, 1999). One of these criteria is diagnosability. Authors should provide 'a statement in words that purports to give those characters which differentiate the taxon from other taxa with which it is likely to be confused' (ICZN, 1999). In this perspective, morphological characters remain as the main criteria for species delimitation in zoological taxonomy (Dantas-Torres, 2018). However, when only a typological-based approach is used for delineating closely related species, *bona fide* species may be considered as a single nominal species due to few or no morphological differences (Dantas-Torres, 2018). Consequently, these 'hidden' cryptic species may not be formally named (Struck *et al.*, 2018).

Over time, groups of organisms previously considered to represent a single 'complex' species (e.g., highly polymorphic, with unclear diagnosability) have been recognized as two or more valid species (e.g., Hebert *et al.*, 2004; Braune *et al.*, 2008; Elmer *et al.*, 2010; Korshunova *et al.*, 2019; Patterson *et al.*, 2020). Examples are available for many groups of arthropods of medical and veterinary significance (e.g., Scarpassa & Alencar, 2012; Coetzee *et al.*, 2013; Nava *et al.*, 2014; Souza *et al.*, 2017). In many instances, the lack of morphological differences has been superseded by broader analyses based on integrative taxonomy (Trontelj & Fier, 2009; Padial *et al.*, 2010; Dantas-Torres, 2018). Eventually, the recognition of cryptic species (including description of new species

and the resurrection of former junior synonyms) has been possible by thorough morphological analysis, which revealed phenetic differences that have been overlooked (Sábio *et al.*, 2014, 2016a; Labruna *et al.*, 2020).

Detecting cryptic species

Besides being important for shedding light on the global biodiversity, detecting cryptic species may be of medical and veterinary interest, when dealing with disease vectors (Bickford *et al.*, 2007; Dantas-Torres, 2018). By definition, cryptic species are detected by genetic analysis (Bickford *et al.*, 2007; Struck *et al.*, 2018). In this regard, numerous molecular markers have been widely used for species diagnosis and evolutionary inferences (Depaquit, 2014). In particular, DNA barcoding approaches normally using mitochondrial genes, such as cytochrome *c* oxidase I (*cox*1) and 16S ribosomal RNA (*16S rRNA*), have been widely applied. As the costs of the DNA sequencing have dropped considerably in recent years, DNA barcoding has increasingly become a cost-effective and accessible approach for large-scale molecular species identification (Hebert *et al.*, 2003), uncovering an extraordinary cryptic diversity in various groups of arthropods (Hebert *et al.*, 2004; Witt *et al.*, 2006; Ratnasingham & Hebert, 2013), including biting midges, phlebotomine sand flies and ticks (Dantas-torres *et al.*, 2013; Pinto *et al.*, 2015; Gajapathy *et al.*, 2016; Erisoz Kasap *et al.*, 2019; Mignotte *et al.*, 2020). Nevertheless, delimiting cryptic species based solely on partial sequences of a mitochondrial barcoding gene may not be reliable (e.g., Vyskočilová *et al.*, 2018). For instance, *cox*1 analyses did not distinguish the ticks *Hyalomma dromedarii* Koch or *Hyalomma truncatum* Koch from *Hyalomma rufipes* Koch collected on camels (Rees *et al.*, 2003). Similarly, pairwise *16S rRNA* gene analysis showed a genetic similarity 98–99% between *Ixodes ricinus* (Linnaeus) and *Ixodes inopinatus* Estrada-Peña, Nava and Petney, which are valid species distinguishable morphologically (Estrada-Peña *et al.*, 2014; Dantas-Torres, 2018). Also, *cox*1 was unreliable for distinguishing Argentinian populations of the mosquitoes *Culex pipiens* Linnaeus and *Culex quinquefasciatus* Say (Laurito *et al.*, 2013). As far as phlebotomine sand flies, in a recent study, the authors were unable to distinguish *Evandromyia carmelinoi* (Ryan, Fraiha, Lainson & Shaw), *Evandromyia evandroi* (Costa Lima & Antunes) and *Evandromyia lenti* (Mangabeira) using *cox*1, even considering their clear morphological differences (Rodrigues *et al.*, 2020). In fact, evolutionary phenomena such as introgression, paternal leakage, heteroplasmy, retention of ancestral polymorphism and/or low interspecific divergence (Rees *et al.*, 2003; Laurito *et al.*, 2013; Pinto *et al.*, 2015; Porretta *et al.*, 2016; Rodrigues *et al.*, 2018; Mastrantonio *et al.*, 2019), should be considered when using DNA-based approaches.

Nuclear genes may also be powerful targets for detecting cryptic species. Particularly, genes controlling aspects associated to the organism fitness (e.g., circadian clock and reproductive behaviour) were primary studied in *Drosophila melanogaster* Meigen (Gleason, 2005). Males of this fly produce songs flapping their wings during the courtship. In particular, two genes are involved in the song rhythm control, namely, *cacophony* and *period* (Gleason, 2005). Mutations in these genes are associated to variations in the song, which act in reproductive recognition, and, in turn, reproductive isolation between *Drosophila* closely related species (Markow & O'Grady, 2005). Homologues of these two genes are found in phlebotomine sand flies and have been applied to assess putative cryptic species (Oliveira *et al.*, 2001; Mazzoni *et al.*, 2002; Bauzer *et al.*, 2002a, 2002b; Bottecchia *et al.*, 2004; Souza *et al.*, 2004; Araki *et al.*, 2009; Salomón *et al.*, 2010; Vigoder *et al.*, 2010; Lima Costa *et al.*, 2015; de Souza Freitas *et al.*, 2016; Souza *et al.*, 2017). The same is true for the *paralytic*, a gene associated with both courtship song and insecticide resistance (Lins *et al.*, 2008).

The abovementioned genes are effective markers for the identification of cryptic species. Multi-locus analyses using mitochondrial genes, nuclear genes, or both, provide a more robust dataset for detecting cryptic species (Araki *et al.*, 2013; Bourke *et al.*, 2013). For instance, multi-locus analyses have been congruent in indicating the existence of cryptic species under the name *Lutzomyia longipalpis* (Lutz & Neiva) in Brazil (Araki *et al.*, 2013).

Microsatellites are also a robust approach for assessing cryptic species. These markers are simple sequence repeats, highly polymorphic and with a high mutation rate (Ellegren, 2004). Microsatellites have been applied for evolutionary studies as well as investigating cryptic diversity in various groups of disease vectors (Gourbière *et al.*, 2012; Depaquit, 2014; Wilke *et al.*, 2014; Araya-Anchetta *et al.*, 2015; Scarpassa *et al.*, 2016; Dorn *et al.*, 2017). Panels with several loci are available for phlebotomine sand fly species from both New and Old Worlds (Day & Ready, 1999; Aransay *et al.*, 2001; Watts *et al.*, 2002, 2005; Santos *et al.*, 2013; Neal *et al.*, 2016; Hamarsheh *et al.*, 2018). In particular, studies using microsatellite revealed the existence of different taxa under the name *Lu. longipalpis* in the Neotropical region (Maingon *et al.*, 2003; Watts *et al.*, 2005; Santos *et al.*, 2013).

Alternatively, non-DNA-based approaches are also powerful tools for assessing cryptic species, such as isoenzymes and matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF MS). Regarding phlebotomine sand flies, isoenzymes have been widely used for investigating taxonomic problems since the 1970s [a rich list of references is available in Depaquit, 2014]. Several studies made use of isoenzymes for investigating the *Lu. longipalpis* species complex (Lanzaro *et al.*, 1993, 1998; Dujardin *et al.*, 1997; Arrivillaga *et al.*, 2003). Le Pont *et al.* (1986) applied isoenzyme on Bolivian sympatric populations of *Psychodopygus carrerai* (Barretto) with a slight morphological difference in mesonotum colouration. This analysis provided evidence for a formal description of *Psychodopygus yucumensis* (Le Pont, Caillard, Tibayrenc & Desjeux) (Caillard *et al.*, 1986). In contrast, similar analyses did not distinguish specimens of *Psychodopygus wellcomei* Fraiha, Shaw & Lainson and *Psychodopygus complexus* (Mangabeira) (Ready & Da Silva, 1984), whose females are morphologically identical (Galati, 2018).

More recently, MALDI-TOF MS appears as a robust method for accurate molecular identification and entomological surveillance (Yssouf *et al.*, 2016; Rakotonirina *et al.*, 2020). This

approach has been successfully applied for some groups of disease vectors (Kaufmann *et al.*, 2011; Yssouf *et al.*, 2013a, 2014; Mewara *et al.*, 2018; Zurita *et al.*, 2019; dos Santos Souza *et al.*, 2020; Gittens *et al.*, 2020; Ouarti *et al.*, 2020; Rot *et al.*, 2020), for discriminating species complexes and cryptic species (Kaufmann *et al.*, 2012; Müller *et al.*, 2013; Yssouf *et al.*, 2013b). Some studies have applied this method for phlebotomine sand fly research (Dvořák *et al.*, 2014, 2020; Mathis *et al.*, 2015; Lafri *et al.*, 2016; Halada *et al.*, 2018a, 2018b; Arfuso *et al.*, 2019) and few ones have focused on New World phlebotomine sand flies (Mathis *et al.*, 2015; Chavy *et al.*, 2019). Yet, no specific study on cryptic species in phlebotomine sand flies was carried out approaching MALDI-TOF MS so far.

Molecular data have been used in conjunction with other lines of evidence to assess cryptic species in phlebotomine sand flies (Souza *et al.*, 2004; Araki *et al.*, 2009;Vigoder *et al.*, 2013, 2015; Spiegel *et al.*, 2016; Souza *et al.*, 2017). For instance, it is acknowledged that the males of different populations of *Lu. longipalpis* produce different types of sex pheromones and courtship songs (Vigoder *et al.*, 2013, 2015; Spiegel *et al.*, 2016; Souza *et al.*, 2017). Recently, it has been demonstrated that the type courtship song plays a role in the *Lu. longipalpis* female's insemination success (Vigoder *et al.*, 2020). These sexual behaviours have been associated to partial or complete reproductive isolation between populations of *Lu. longipalpis* (Ward *et al.*, 1988; Souza *et al.*, 2017).

While detecting cryptic species may be relatively easy when a comprehensive integrative approach is employed, it is not always a simple task to name a cryptic species, even when different lines of evidence are available (Brandão-Filho *et al.*, 2009; Dantas-Torres & Otranto, 2015).

Naming cryptic species

Diagnosis is one of the criteria required for naming new species in zoological taxonomy (ICZN, 1999). Since 1st January 1758 (i.e., the starting point of zoological nomenclature), morphological characters have been the basis for the description of the absolute majority of animal species on Earth. Considering that, by definition, two cryptic species are morphologically indistinguishable (some authors have considered species with minor morphological differences as cryptic species, but this conceptual discussion is beyond the scope of this article), they cannot be delineated based on morphological characters. As such, other criteria (e.g., fixed single nucleotide polymorphisms – SNPs) should be used to delineate a cryptic species, as recently done with ticks (Bakkes *et al.*, 2018). Nonetheless, caution should be exercised when using single-gene datasets, from a small number of samples (Dantas-Torres, 2018). Conversely, when a reliable number of specimens are analysed, fixed-SNPs may support interspecific variations between putative cryptic species (Lima Costa *et al.*, 2015).

A formal description of a cryptic species should follow strictly the rules of the International Code of on Zoological Nomenclature (ICZN, 1999). A pivotal step while delineating a cryptic species is defining which taxon should be maintained as the sensu stricto species. Although this may be relatively easy when genetic data from the name-bearing type (e.g., holotype) are available, additional efforts may be required when the type specimen for the sensu stricto species is unavailable, including the designation of a neotype. This is the case of phlebotomine sand flies currently identified as *Lu. longipalpis*, which clearly included two or more cryptic species, but a name-bearing specimen is unavailable (Brandão-Filho *et al.*, 2009). Thus, in this case, a neotype should be designated, as recently done with *Rhipicephalus sanguineus* (Latreille), the brown dog tick (Nava *et al.*, 2018).

Species complexes and cryptic species in New World phlebotomine sand flies

Until recently, American phlebotomine sand flies were grouped into three genera (i.e., *Lutzomyia* França, *Brumptomyia* França & Parrot and *Warileya* Hertig) (Young & Duncan, 1994), with all vector species included in the genus *Lutzomyia*. In the past 25 years, several changes in the classification and nomenclature of phlebotomine sand flies have been proposed (Galati, 2018). To date, more than 540 phlebotomine sand fly species belonging to 23 genera are recognized as valid in the Americas (Shimabukuro *et al.*, 2017; Galati, 2018). Of these, 158 (~30%) show some taxonomic problems, i.e., male, female, or both, of some species are indistinguishable morphologically (Table 1). Notably, some of these indistinguishable females belong to species [e.g., *Lu. longipalpis*, *Lutzomyia cruzi* (Mangabeira), *Ps. complexus*, *Ps. wellcomei*] that have been previously reported as proven or putative vector of *Leishmania* spp. (Maroli *et al.*, 2013; Brazil *et al.*, 2015). In this section, we point out the American phlebotomine sand flies that comprise species complexes whose members have been proven or putatively associated with pathogen transmission.

A classic example is *Lu. longipalpis*, the main vector of *Leishmania infantum* Nicolle, the causative agent of visceral leishmaniasis in the American continent (Sousa-Paula *et al.*, 2020). Different lines of evidence are congruent in indicating that the name '*Lu. longipalpis*' presently includes an uncertain number of cryptic species (reviewed by Spiegel *et al.*, 2016 and Souza *et al.*, 2017). In addition to molecular data, these cryptic species have been assessed by crossbreeding experiments as well as analyses of courtship songs and sex pheromones produced by males (Souza *et al.*, 2008; Araki *et al.*, 2009, 2013; Salomón *et al.*, 2010; Lima Costa *et al.*, 2015; Vigoder *et al.*, 2015; Pech-May *et al.*, 2018). Yet, no formal description has been proposed so far (Brandão-Filho *et al.*, 2009; Souza *et al.*, 2017).

In addition, it is acknowledged that the *Lu. longipalpis* complex also includes the closely related species *Lu. cruzi* and *Lutzomyia pseudolongipalpis* Arrivillaga & Feliciangeli (Arrivillaga & Feliciangeli, 2001; Watts *et al.*, 2005; Vigoder *et al.*, 2010). *Lutzomyia cruzi* is also reputed to be a vector of *L. infantum* in the centre-west region of Brazil (Vigoder *et al.*, 2010; Falcão de Oliveira *et al.*, 2017). Females of *Lu. longipalpis* and *Lu. cruzi* are morphologically indistinguishable (Mangabeira Filho, 1969; Giordani *et al.*, 2017), but males can be differentiated. *Lutzomyia pseudolongipalpis* is reported as vector of *L. infantum* in Venezuela and the male

Table 1. American phlebotomine sand flies (Diptera: Psychodidae: Phlebotomine), whose females, males, or both, are morphologically indistinguishable. Pathogens provenly or putatively transmitted by them are indicated.

Group‡	Genus (subgenus)	Species*	Sex	Pathogens provenly or putatively transmitted†
1	*Brumptomyia*	*Br. mesai*	F	
		Br. pentacantha	F	
2	*Bichromomyia*	*Bi. flaviscutellata*	M	*L. amazonensis*
		Bi. inornata	M	
3	*Deanemyia*	*De. derelicta*	F	
		De. ramirezi	F	
	Micropygomyia			
4	(*Sauromyia*)	*Mi. quinquefer*	F	
		Mi. zikani	F	
5	(*Micropygomyia*)	*Mi. cayennensis*	M	
		Mi. cubensis	M	
		Mi. hardisoni	M	
6		*Mi. cayennensis cayennensis*	F	
		Mi. farilli	F	
		Mi. hardisoni	F	
7		*Mi. chassigneti*	F	
		Mi. mangabeirana	F	
		Mi. pilosa	F	
	Lutzomyia			
8	(*Helcocyrtomyia*)	*Lu. gonzaloi*	F	
		Lu. hartmanni	F	*L. panamensis*, *L. colombiensis*
		Lu. kirigetiensis	F	
		Lu. tortura	F	
9		*Lu. guderiani*	F	
		Lu. monzonensis	F	
10		*Lu. scorzai*	F	
		Lu. tolimensis	F	
11		*Lu. erwindonaldoi*	M	
		Lu. larensis	M	
12		*Lu. galatiae*	F	
		Lu. tejadai	F	*L. braziliensis*
13	(*Lutzomyia*)	*Lu. longipalpis*	M	*L. infantum*, *L. amazonensis*
		Lu. pseudolongipalpis	M	*L. infantum*
14		*Lu. battistinii*	F	
		Lu. bicornuta	F	
		Lu. ischnacantha	F	
15		*Lu. cruzi*	F	*L. infantum*
		Lu. gaminarai	F	
		Lu. longipalpis	F	*L. infantum*, *L. amazonensis*
		Lu. matiasi	F	
16	(*Tricholateralis*)	*Lu. carvalhoi*	F	
		Lu. falcata	F	
		Lu. maesi	F	
		Lu. marinkellei	F	
		Lu. spathotrichia	F	
	Pintomyia			
17	(*Pintomyia*)	*Pi. christenseni*	F	
		Pi. damascenoi	F	
18		*Pi. fischeri*	F	*L. braziliensis*
		Pi. gibsoni	F	
19	(*Pifanomyia*)	*Pi. andina*	F	
		Pi. aulari	F	
20		*Pi. moralesi*	F	
		Pi. verrucarum	F	*B. bacilliformis*, *L. peruviana*
21		*Pi. odax*	F	
		Pi. ottolinai	F	
		Pi. robusta	F	*B. bacilliformis*
		Pi. serrana	F	*B. bacilliformis*
22		*Pi. amilcar*	F	
		Pi. longiflocosa	F	*L. guyanensis*
		Pi. nadiae	F	
		Pi. quasitownsendi	F	
		Pi. sauroida	F	
		Pi. spinicrassa	F	*L. mexicana*, *L. braziliensis*
		Pi. torvida	F	
		Pi. townsendi	F	*L. braziliensis*
		Pi. youngi	F	*L. mexicana*, *L. garnhami*, *L. braziliensis*
	Dampfomyia			
23	(*Dampfomyia*)	*Da. anthophora*	F	*L. mexicana*
		Da. dodgei	F	
24		*Da. atulapai*	F	
		Da. rosabali	F	
25	Delpozoi group	*Da. delpozoi*	F	
		Da. inusitata	F	
26	(*Coromyia*)	*Da. deleoni*	F	
		Da. vesicifera	F	
		Da. zeledoni	F	
27		*Da. beltrani*	F	
		Da. disneyi	F	
		Da. steatopyga	F	
28	*Pressatia*	*Pr. calcarata*	F	
		Pr. camposi	F	
		Pr. choti	F	*L. brazilienesis*
		Pr. duncanae	F	
		Pr. dysponeta	F	
		Pr. equatorialis	F	
		Pr. triacantha	F	
		Pr. trispinosa	F	
29	*Trichopygomyia*	*Ty. longispina*	M	
		Ty. ratcliffei	M	
30		*Ty. conviti*	F	
		Ty. dasypodogeton	F	
		Ty. depaquiti	F	
		Ty. elegans	F	
		Ty. ferroae	F	
		Ty. gantier	F	
		Ty. longispina	F	*L. braziliensis*
		Ty. martinezi	F	
		Ty. pinna	F	
		Ty. ratcliffei	F	
		Ty. rondoniensis	F	
		Ty. trichopyga	F	
		Ty. triramula	F	
		Ty. turelli	F	
		Ty. wagley	F	
		Ty. witoto	F	
	Evandromyia			
31	(*Evandromyia*)	*E. bourrouli*	F	
		Ev. pinottii	F	
32	(*Barrettomyia*)	*Ev. cortelezzii*	F	
		Ev. sallesi	F	*L. infantum*
33		*Ev. corumbaensis*	F	
		Ev. spelunca	F	
34	Tupynambai series	*Ev. bahiensis*	F	
		Ev. callipyga	F	
		Ev. costalimai	F	
		Ev. petropolitana	F	
		Ev. tupynambai	F	

Table 1. Continued

Group‡	Genus (subgenus)	Species*	Sex	Pathogens provenly or putatively transmitted†
	Psathyromyia			
35	(*Forattiniella*)	*Pa. inflata*	F	
		Pa. runoides	F	
36	(*Xiphopsathyromyia*)	*Pa. dreisbachi*	F	
		Pa. hermanlenti	F	
		Pa. ruparupa	F	
37	(*Psathyromyia*)	*Pa. lanei*	F	
		Pa. pelloni	F	
38		*Pa. cratifer*	F	
		Pa. limai	F	
39		*Pa. ribeirensis*	F	
		Pa. undulata	F	
40	*Viannamyia*	*Vi. caprina*	F	
		Vi. furcata	F	
41	*Psychodopygus*	*Ps. chagasi*	F	
		Ps. complexus	F	*L. braziliensis*
		Ps. douradoi	F	
		Ps. fairtigi	F	
		Ps. killicki	F	
		Ps. squamiventris	F	*L. naiffi*
		Ps. squamiventris maripaensis	F	
		Ps. wellcomei	F	*L. braziliensis*
42		*Ps. corossoniensis*	F	
		Ps. francoisleponti	F	
		Ps. geniculatus	F	
		Ps. guyanensis	F	
43	*Nyssomyia*	*Ny. antunesi*	F	*L. lindenbergi*
		Ny. urbinattii	F	
44		*Ny. fraihai*	F	
		Ny. yuilli yuilli	F	
45	*Trichophoromyia*	*Th. adelsonsouzai*	F	
		Th. auraensis	F	*L. braziliensis*, *L. lainsoni*, *L. guyanensis*
		Th. beniensis	F	
		Th. bettinii	F	
		Th. brachipyga	F	*L. lainsoni*
		Th. castanheirai	F	
		Th. eurypyga	F	
		Th. howardi	F	
		Th. ininii	F	*L. braziliensis*
		Th. napoensis	F	
		Th. pabloi	F	
		Th. pastazaensis	F	
		Th. readyi	F	
		Th. rostrans	F	
		Th. ruii	F	
		Th. ruifreitasi	F	*L. braziliensis*, *L. guyanensis*
		Th. velascoi	F	
		Th. velezbernali	F	
		Th. viannamartinsi	F	
		Th. wilkersoni	F	

*Largely based on Galati (2018).

†Largely based on Maroli *et al.* (2013) and Brazil *et al.* (2015).

‡Groups of species, whose females, males, or both, are morphologically indistinguishable.

L., *Leishmania*; B., *Bartonella*; F, Female; M, Male.

of this species is indistinguishable from that of *Lu. longipalpis* (Agrela & Feliciangeli, 2015; Galati, 2018). *Lutzomyia gaminarai* (Cordero, Vogelsang & Cossio), whose female is morphologically indistinguishable from that of *Lu. longipalpis*, should also be included in the *Lu. longipalpis* complex (Bauzer *et al.*, 2007). The same is true for *Lutzomyia matiasi* Le Pont & Mollinedo. Furthermore, while males and females of *Lutzomyia alencari* Martins, Souza & Falcão can be differentiated using morphological traits from those of *Lu. longipalpis* (Galati, 2018), genetic data indicate that *Lu. alencari* should be included in the *Lu. longipalpis* complex as well (Pinto *et al.*, 2015).

Nyssomyia umbratilis (Ward & Fraiha) is the main vector of *Leishmania guyanensis* Floch in the Amazonian region. Studies suggested that there may be at least two cryptic species under the name *Ny. umbratilis* (Scarpassa & Alencar, 2012), which are presently not formally recognized. Interestingly, population structuring and phylogenetic data have shown that the Brazilian populations from Rio Preto da Eva (north of the Negro River; Amazonas state) and Recife (Pernambuco state) comprise a different clade when compared to a population from Manacapuru (south of the Negro River; Amazonas state) (de Souza Freitas *et al.*, 2015, 2016). Furthermore, some minor differences in the armature of genital atrium between specimens from Rio Preto da Eva and Manacapuru have been reported as well (Farias *et al.*, 2015). Yet, no formal description has been proposed for this putative species. More recently, an in vitro study demonstrated that the Manacapuru population of *Ny. umbratilis* is refractory to *L. guyanensis* attachment on the midgut (Soares *et al.*, 2018). This suggests a direct difference in vector competence between two of these putative cryptic species.

The *Psathyromyia shannoni* (Dyar) complex includes vectors of *Vesiculovirus*, the causative agent of vesicular stomatitis, and putative vectors of *Leishmania* spp. (Travi *et al.*, 2002; Pech-May *et al.*, 2010; Maroli *et al.*, 2013). Until recently, *Pa. shannoni* was considered the most widespread phlebotomine sand fly species in the Americas, ranging from the United States to Argentina (Young & Duncan, 1994; Galati, 2003). However, a taxonomic review based on morphological and morphometric characters (Sábio *et al.*, 2014, 2016a, 2016b) resulted in the resurrection of *Psathyromyia bigeniculata* (Floch & Abonnenc), *Psathyromyia limai* (Fonseca) and *Psathyromyia pifanoi* (Ortiz), which were previously considered to be junior synonyms of *Pa. shannoni*. This comprehensive work also led to the description of new species, namely, *Psathyromyia ribeirensis* Sábio, Andrade & Galati and *Psathyromyia baratai* Sábio, Andrade & Galati. Another member of this complex is *Psathyromyia abonnenci* (Floch & Chassignet). While females of *Pa. limai* and *Pa. ribeirensis* are morphologically indistinguishable (Galati, 2018), other females from species belonging to the *Pa. shannoni* complex can be differentiated by a trained taxonomist.

Bichromomyia genus comprises six species, of which five compose the *Bichromomyia flaviscutellata* complex (Galati, 2018; de Melo *et al.*, 2020). Among them, *Bichromomyia flaviscutellata* (Mangabeira) is considered the main vector of *Leishmania amazonensis* Lainson & Shaw in Brazil (Brazil *et al.*, 2015). Furthermore, *Bichromomyia reducta* (Feliciangeli, Ramirez Pérez & Ramirez), *Bichromomyia olmeca olmeca* (Vargas & Díaz-Nájera), *Bichromomyia olmeca bicolor* (Fairchild & Theodor) and *Bichromomyia olmeca nociva* (Young & Arias) are vectors of *Leishmania* spp. as well (Maroli *et al.*, 2013).

Psychodopygus wellcomei is an important vector of *Leishmania braziliensis* Vianna, the most prevalent causative agent of

cutaneous leishmaniasis in the Americas (Brazil *et al.*, 2015). Females of *Ps. wellcomei* are indistinguishable from those of *Ps. complexus*, which is also a vector of *L. braziliensis* (Maroli *et al.*, 2013; Brazil *et al.*, 2015). These species may occur in sympatry in forested areas, as demonstrated in a military training camp where cutaneous leishmaniasis outbreaks have been reported (Dantas-Torres *et al.*, 2017; Da Silva *et al.*, 2019). Moreover, these two species compose a morphologically indistinguishable group of females with other six species (Table 1), including *Psychodopygus squamiventris* (Lutz & Neiva), a putative vector of *Leishmania naiffi* Lainson & Shaw (Maroli *et al.*, 2013).

Pintomyia Costa Lima is another genus with some indistinguishable females. For instance, the so-called 'Verrucarum series' (*sensu* Fairchild, 1955) is a species complex which currently includes *Pintomyia verrucarum* (Townsend) and other nine related species, whose females are sometimes indistinguishable [*Pintomyia aulari* (Feliciangeli, Ordoñez & Manzanilla) from *Pintomyia andina* (Osorno, Osorno-Mesa & Morales) and *Pintomyia moralesi* (Young) from *Pi. verrucarum*] or virtually unknown [*Pintomyia antioquiensis* Wolff & Galati, *Pintomyia deorsa* (Pérez, Ogusuku, Monje & Young) and *Pintomyia itza* Ibáñez-Bernal, May-UC & Rebollar-Tellez)]. *Pintomyia verrucarum* is an important vector of *Bartonella bacilliformis*, a bacterium that causes Carrión's disease in Andean region (Sanchez Clemente *et al.*, 2012; Battisti *et al.*, 2015; Garcia-Quintanilla *et al.*, 2019). Moreover, this phlebotomine sand fly is a known vector of *Leishmania peruviana* Velez in the Peruvian Andes (Maroli *et al.*, 2013; Brazil *et al.*, 2015). Another species complex within the genus *Pintomyia* is the 'Townsendi Series' (*sensu* Galati, 2003). Among others, this complex includes *Pintomyia spinicrassa* (Morales, Osorno-Mesa, Osorno & Hoyos), *Pintomyia longiflocosa* (Osorno-Mesa, Morales, Osorno & Hoyos), *Pintomyia townsendi* (Ortiz) and *Pintomyia youngi* (Feliciangeli & Murillo), which are proven or putative vectors of *Leishmania* spp. (Maroli *et al.*, 2013).

Females belonging to the genus *Trichophoromyia* Barretto are generally indistinguishable (20 species) (Table 1) or very difficult to identify based on morphology (Galati, 2018; Posada-López *et al.*, 2018; Dos Santos & Silveira, 2020); several females of this genus are unknown (Galati, 2018). This group includes *Trichophoromyia ubiquitalis* (Mangabeira), the main vector of *Leishmania lainsoni* Silveira, Shaw, Braga & Ishikawa in Pará state, Brazil (Maroli *et al.*, 2013; Brazil *et al.*, 2015). In addition, other closely related species, such as *Trichophoromyia auraensis* (Mangabeira), *Trichophoromyia brachipyga* (Mangabeira), *Trichophoromyia ininii* (Floch & Abonnenc), *Trichophoromyia ruifreitasi* Oliveira, Teles, Medeiros, Camargo & Pessoa and *Trichophoromyia velascoi* (Le Pont & Desjeux) have been suggested as vectors of *Leishmania* spp. as well (Valdivia *et al.*, 2012; Brazil *et al.*, 2015; Teles *et al.*, 2016; De Araujo-Pereira *et al.*, 2017; Dos Santos & Silveira, 2020).

The genus *Evandromyia* Mangabeira includes at least six groups of species (Galati, 2018). Among these, the *Evandromyia cortelezzii* (Brèthes) complex [Cortelezzii series, *sensu* Galati, 2003] encompasses a group of five morphologically similar species, which are widespread in Latin America (Carvalho *et al.*, 2009; Galati, 2018). There are no morphological traits for discriminating females of *Ev. cortelezzii* and *Evandromyia sallesi* (Galvão & Coutinho). The same is true for females of *Evandromyia corumbaensis* (Galati, Nunes, Oshiro & Rego) and *Evandromyia spelunca* Carvalho, Brazil, Sanguinette & Andrade Filho. By contrast, females of *Evandromyia chacuensis* Szelag, Rosa, Galati, Andrade Filho & Salomón are distinguishable from those species of the complex (Galati, 2018). Morphologically, *Ev. chacuensis* is closer to *Ev. corumbaensis* and *Ev. spelunca* than to *Ev. cortelezzii* and *Ev. sallesi* (Szelag *et al.*, 2018). For this reason, it has been suggested to separate the *Ev. cortelezzii* complex, with *Ev. corumbaensis*, *Ev. spelunca* and *Ev. chacuensis* comprising a new species complex, the *corumbaensis* complex (Szelag *et al.*, 2018). There has been reported natural *Leishmania* spp. infections in *Ev. sallesi* and *Ev. cortelezzii* through midgut dissection and/or molecular tools (Carvalho *et al.*, 2008; Saraiva *et al.*, 2009; Rosa *et al.*, 2012). These findings suggest that members of the *Ev. cortelezzii* complex may be involved in the transmission of *Leishmania* spp.

Dampfomyia (Addis) is another genus with several species presenting indistinguishable females (Table 1). As far as epidemiological importance, *Dampfomyia anthophora* (Addis) have been associated with the transmission of *Leishmania mexicana* Biagi in the US (Endris *et al.*, 1987; McHugh *et al.*, 2001). Females of *Da. anthophora* are morphologically identical to *Dampfomyia dodgei* (Vargas & Diaz-Nájera).

The abovementioned examples illustrate how problematical may be the taxonomy of phlebotomine sand flies, especially when based solely on morphology. The inability to identify one or more species may have pragmatic implications, especially when dealing with species potentially involved in the transmission of pathogens.

Practical implications of species complexes and cryptic species of New World phlebotomine sand flies

The taxonomic interest on many species complexes of arthropod vectors has improved our knowledge on these groups, especially from an evolutive viewpoint (Harbach, 2012; Araki *et al.*, 2013; Fontaine *et al.*, 2015). On the other hand, studies focusing on cryptic species and their roles as vectors of disease agents are limited. This is remarkably apparent for neglected vectors, such as biting midges, black flies, triatomine bugs as well as phlebotomine sand flies.

The *Lu. longipalpis* complex is presently one of the most well-studied group among American phlebotomine sand flies. A pioneer study suggested that reproductive isolated populations may act differently in the transmission of *L. infantum* in Brazil (Ward *et al.*, 1983). Thereafter, conclusive studies demonstrated that populations of *Lu. longipalpis* from Costa Rica, Colombia and Brazil (Lanzaro *et al.*, 1993) can differently modulate the leishmaniasis imunopathogenicity (Warburg *et al.*, 1994; Lanzaro *et al.*, 1999; Yin *et al.*, 2000). Studies based on sex pheromone analyses to identify *Lu. longipalpis* putative cryptic species have suggested that some chemotype populations (i.e., a given population producing a certain sex pheromone type

[reviewed in Spiegel *et al.*, 2016]) could have different vector capacity for *L. infantum* transmission (Casanova *et al.*, 2006, 2015). In São Paulo state, Brazil, it has been suggested that the (S)-9-methylgermacrene-B chemotype population has a greater vector capacity as compared with the cembrene-1 chemotype population. However, the cembrene-1 chemotype population has been recorded in other Brazilian regions where visceral leishmaniasis is highly prevalent (Hamilton *et al.*, 2005; Casanova *et al.*, 2006, 2015). In Brazil, visceral leishmaniasis control have failed in part due to limited financial resources available for vector control and other strategies (Dantas-Torres *et al.*, 2019; Sousa-Paula *et al.*, 2019). Studies investigating the vector capacity of putative cryptic species belonging to the *Lu. longipalpis* complex may be helpful for optimizing vector control strategies.

Nyssomyia umbratilis is another example of different vector role played by two putative cryptic species (Fig. 1). As mentioned previously, the population from Manacapuru is refractory to in vitro interaction with *L. guyanensis* (Soares *et al.*, 2018). It is interesting to note that it has long been reported that *L. guyanensis* is absent in the south of Negro River, where this population is found (Arias & Freitas, 1978; Scarpassa & Alencar, 2012). Although there is solid circumstantial evidence suggesting *Ny. umbratilis* from Manacapuru may not transmit *L. guyanensis*, no experimental transmission studies have been performed to date. This may be partly explained by the difficulties in establishing a laboratory colony of *Ny. umbratilis* (F. A. C. Pessoa, personal observations).

It is interesting to note that populations of *Ny. umbratilis* recorded outside the Amazonian region are abundant in Atlantic forest remnants (Balbino *et al.*, 2001, 2005a, 2005b). While these populations belong to a clade that includes a *L. guyanensis*-vector population from Rio Preto da Eva (de Souza Freitas *et al.*, 2015, 2016), the role of *Ny. umbratilis* as vector outside the Amazonian region remains unproven. More recently, *Ny. umbratilis* has been recorded in other non-Amazonian areas from Brazil (de Souza Freitas *et al.*, 2018). Further studies should be carried out to elucidate the taxonomy of the non-Amazonian populations of *Ny. umbratilis* and if they may act in the transmission of *L. guyanensis* or other *Leishmania* spp.

The above examples underline that studying species complexes and eventually naming cryptic species is of practical interest. Examples are also available among Old World phlebotomine sand flies (e.g., Ilango, 2010; Gajapathy *et al.*, 2013; Kasap *et al.*, 2013; Depaquit *et al.*, 2019), as it is the case of *Phlebotomus chabaudi* Croset, Abonnenc & Rioux and *Phlebotomus riouxi* Depaquit, Léger & Killick-Kendrick, putative vectors of *Leishmania* spp. (Lehrter *et al.*, 2017). Although some authors have questioned the validity of *Ph. riouxi* (Tabbabi *et al.*, 2014), other authors have presented morphological and phylogenetic data supporting their separation (Lehrter *et al.*, 2017).

Another relevant example is the *Phlebotomus perniciosus* Newstead complex, whose members may play different roles in *L. infantum* transmission (Pesson *et al.*, 2004; Zarrouk *et al.*, 2016). Similarly, the *Phlebotomus perfiliewi* complex encompass important vectors of leishmaniasis (Depaquit *et al.*, 2013). While *Ph. perfiliewi perfiliewi* (Parrot) and *Phlebotomus perfiliewi transcaucasicus* (Perfiliev) have been associated with *L. infantum* transmission (Rassi *et al.*, 2009; Depaquit *et al.*, 2013), the role of *Phlebotomus perfiliewi galilaeus* (Theodor) is still unknown (Depaquit *et al.*, 2013).

Concluding remarks and future research

Beyond taxonomy, it is fundamental to detect and to delineate cryptic species within a given species complex, especially when dealing with certain groups of arthropods that include recognized vectors of medical and veterinary importance. This is the case of phlebotomine sand flies. Indeed, giving formal names to cryptic species may be important for understanding the transmission dynamics of certain vector-borne diseases, such as leishmaniasis. Nonetheless, caution should be exercised to avoid premature description of cryptic species, in the absence of solid phylogenetic data supporting its separation from its closest relative.

For most of the phlebotomine sand fly species complexes discussed herein, our knowledge of basic biology and vector roles is incipient. Information on natural breeding sites, blood-meal preferences and vector competence to transmit *Leishmania* spp. is fundamental when studying such species complexes from a practical standpoint. Recently developed approaches are useful for investigating these aspects (Sales *et al.*, 2015, 2020; Hlavackova *et al.*, 2019), particularly where different taxa coexist. In the same way, novel *Leishmania* stage-specific markers have been proposed (Coutinho-Abreu *et al.*, 2020) and may be used for prospecting *Leishmania* spp. metacyclic stages in field-caught phlebotomine sand flies, thus providing strong evidence of the participation of a given species as a putative vector. Another important aspect to be investigated is the possibility of generalist species (e.g., catholic feeding behaviour or permissiveness for several pathogens) may represent in reality a cryptic species complex (Kankare *et al.*, 2005; Smith *et al.*, 2006; Bickford *et al.*, 2007; Gómez-Díaz *et al.*, 2010).

As discussed in this article, detecting cryptic species may be relatively easier than naming them. In some cases, primary taxonomic problems, as the lack of type specimens, should be solved prior to the description of any new species. Incidentally, this is the case of *Lu. longipalpis*, whose type specimen is currently unavailable. In such cases, a neotype should be designated.

Understanding the biology, ecology and vector role of the cryptic species of arthropods of medical and veterinary importance is not trivial. Collaborative multicentre studies may be a valuable approach to elucidate the basic aspects, especially when dealing with different lines of evidence. In this perspective, efforts should be done to improve current protocols for mass-rearing, especially for species that have not been colonized at large scale so far (e.g., *Ny. umbratilis*). Laboratory rearing is a difficult task, but it is essential to investigate biological life cycles of both vectors and transmitted pathogens, vector competence and capacity, reproductive isolation and insecticide resistance.

Studies for increasing our knowledge on the global diversity of phlebotomine sand flies and for elucidating their evolutionary relationships are fundamental. In this perspective, comparative studies based on genomics and other -omics, which has been

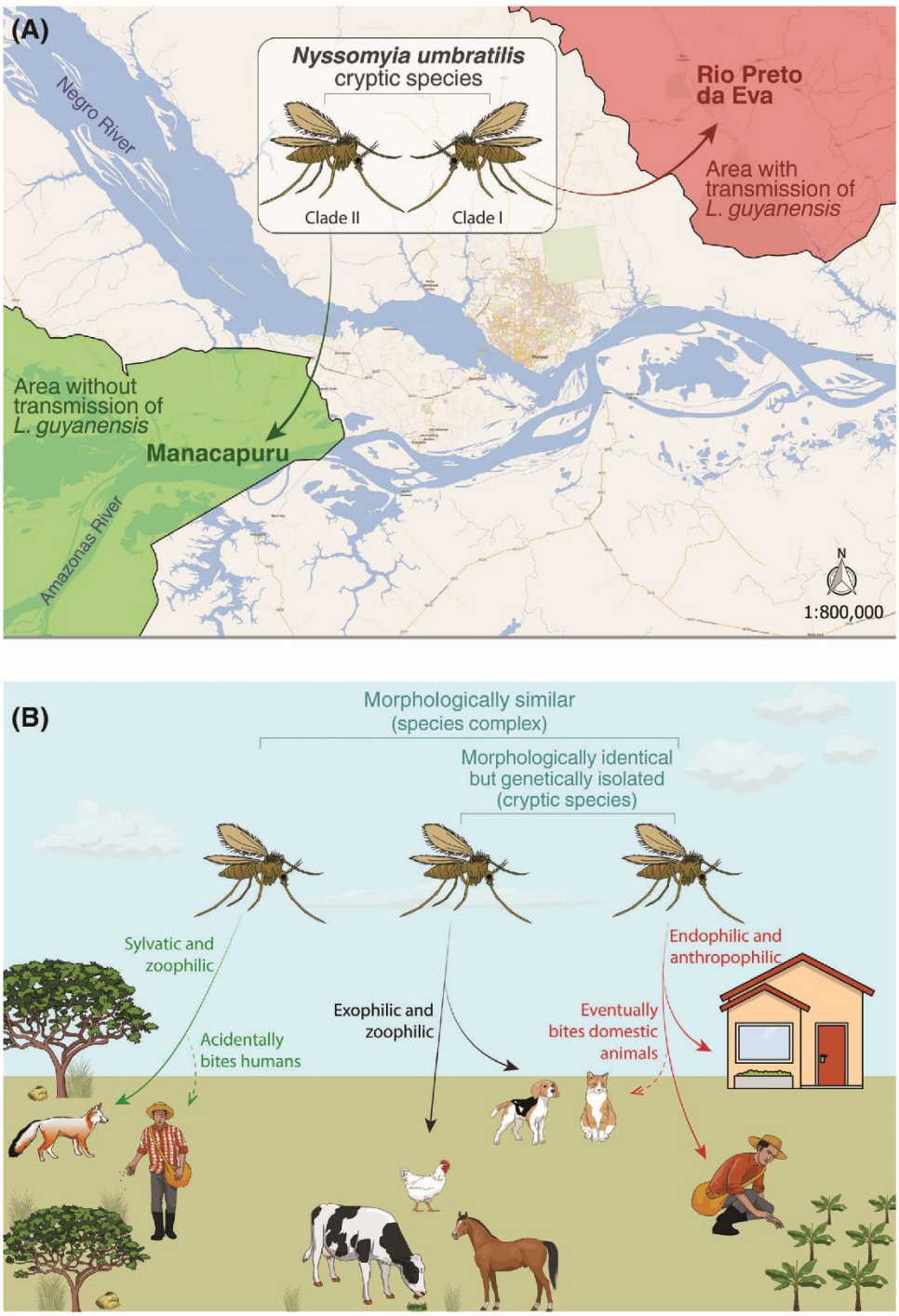

Fig. 1. Phlebotomine sand fly cryptic species playing different roles in *Leishmania* spp. transmission. (A) Populations (clade I and clade II *sensu* Scarpassa & Alencar, 2012) of *Nyssomyia umbratilis* from two localities from Amazonas state (Brazil) putatively display differences in vector competence. In the red area (Rio Preto da Eva), *Leishmania guyanensis* is transmitted by *Ny. umbratilis* clade I, whereas in the green area (Manacapuru) *Ny. umbratilis* clade II apparently is not a vector (for details, see text). (B) A hypothetical scheme showing how members of species complexes may play different roles in the transmission of the vector-borne disease. The colour of the arrows indicates the risk of pathogen transmission (red: high; green: moderate; black: low).

poorly explored in phlebotomine sand flies, may also uncover an extraordinary amount of information, which may allow a better understanding of cryptic speciation and vector competence in this important group of arthropods.

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DATA AVAILABILITY STATEMENT

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

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4.2 Artigo 2 – *Lutzomyia longipalpis* (Sand Fly)

Nesse artigo publicado em *Trends in Parasitology*[2], nós resumimos aspectos pertinentes à espécie *L. longipalpis* s.l., como transmissão de patógenos, medidas de controle e ciclo de vida. Esse foi o primeiro artigo publicado na recém-criada seção *Vector of the Month* da revista e foi destaque no mês de setembro de 2020 pela editora *Cell Press*.

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Trends in Parasitology | Vector of the Month

Lutzomyia longipalpis (Sand Fly)

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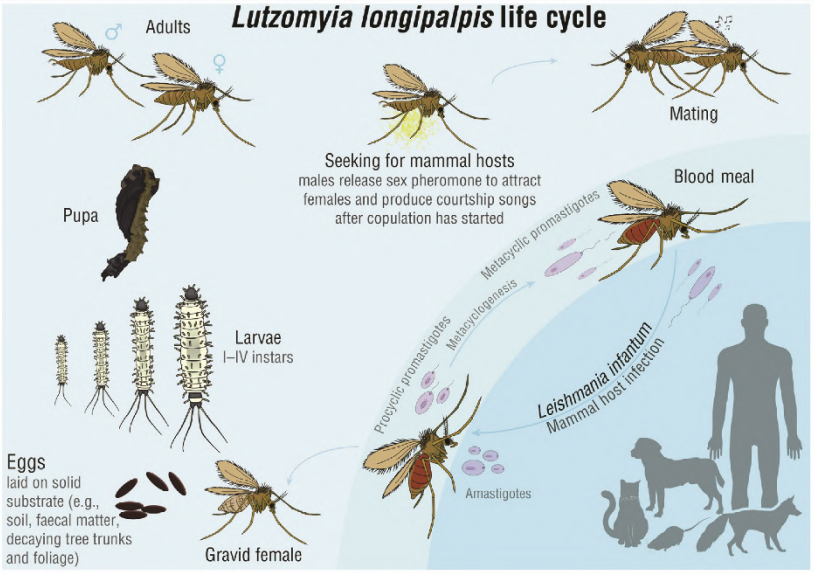
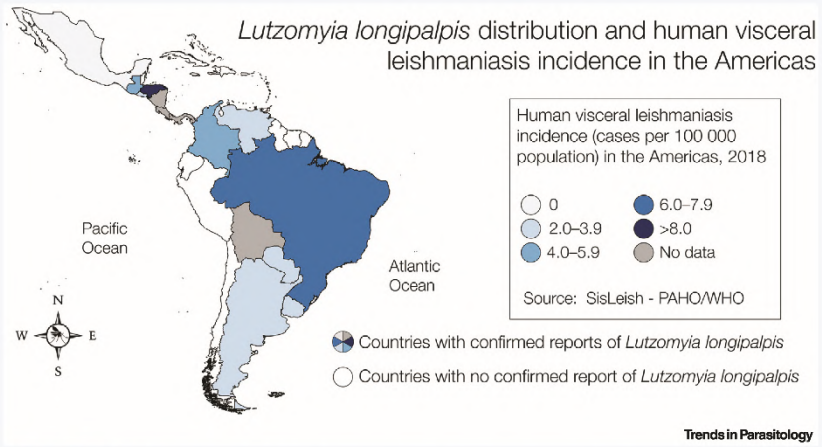


SUMMARY

Lutzomyia longipalpis appears primarily in Central and South America and is the main vector of visceral leishmaniasis (VL) caused by *Leishmania infantum*. In Brazil, the country reporting the highest number of human VL cases in the region, this sand fly is reported in 24 of 27 states. *L. longipalpis* is adapted to human dwellings, which contributes to its spreading in rural and urban areas. Female sand flies are catholic blood feeders with remarkable anthropophilic and endophilic behaviour. The presence of dogs at home and higher dog seropositivity in nearby areas are risk factors for VL. Current control strategies target adult stages. The limited knowledge of *L. longipalpis* breeding sites, which are strictly terrestrial, is a hurdle for controlling the preimaginal stages. In addition, *L. longipalpis* composes a species complex, harbouring an uncertain number of cryptic species. Further research may reveal that some of these cryptic species are more efficient vectors of *L. infantum* than others.



TRANSMISSION FACTS:
L. longipalpis adults may rest in human houses and animal shelters during the day. The biting activity of females is crepuscular and nocturnal.

Sequential blood meals by *L. infantum*-infected *L. longipalpis* females increase infective forms in their gut, potentially augmenting their infectiousness.

L. infantum transmitted by some *L. longipalpis* populations with low amounts of maxadilan (a salivary peptide) may cause cutaneous lesions in Central America.

The sand fly promastigote secretory gel and gut microbiota are egested into host skin during the bite, playing a role in the establishment and visceralization of *Leishmania* infections.

L. longipalpis is widely used as model for experimental transmission, with high biting rate on chicken skin membranes. It is also permissive to several *Leishmania* spp. under laboratory conditions.

CONTROL FACTS:
Insecticide-treated nets and indoor residual spraying can reduce indoor transmission. Both strategies can be boosted when combined with synthetic sex-aggregation pheromones, which attracts both males and females.

Applying topical insecticides (e.g., pyrethroid-based products) on dogs can reduce their exposure to the vectors. The extended use of this strategy in Brazil has not increased *L. longipalpis* insecticide resistance.

TAXONOMY AND CLASSIFICATION:
PHYLUM: Arthropoda
CLASS: Insecta
ORDER: Diptera
FAMILY: Psychodidae
GENUS: *Lutzomyia*
SPECIES: *L. longipalpis* (Lutz and Neiva 1912)

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Resources
http://www.cvbd.org/en/sand-fly-borne-diseases/about-sand-flies/sand-fly-feeding/host-seeking-behaviour/
https://www.who.int/leishmaniasis/disease/vector/en/
https://www.troccap.com/
http://www.leishvet.org/

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4.3 Artigo 3 – Genetic structure of allopatric populations of *Lutzomyia longipalpis* sensu lato in Brazil

Nesse artigo publicado em *Acta Tropica*[3], nós utilizamos ferramentas de bioinformática baseadas em métodos probabilísticos para inferência filogenética e avaliação da estrutura genética de três populações de *L. longipalpis* s.l. colonizadas na Fiocruz Pernambuco. Adicionalmente, nós comparamos nossos dados com os de outras populações de *L*. *longipalpis* s.l. disponíveis em banco de dados públicos.

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Genetic structure of allopatric populations of *Lutzomyia longipalpis* sensu lato in Brazil

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ABSTRACT

Lutzomyia longipalpis sensu lato is a complex of phlebotomine sand fly species, which are widespread in the Neotropics. They have a great medico-veterinary importance due their role as vectors of *Leishmania infantum*, the causative agent of visceral leishmaniasis. Morphological variations of *Lu. longipalpis* s.l. males were reported in the late 1960s in Brazil. Male populations can present either one pair of spots on third abdominal tergites or two pairs on third and fourth ones, namely 1S and 2S phenotypes, respectively. Since then, there has been much interest on the taxonomic status of *Lu. longipalpis* s.l. Thereafter, several lines of evidence have been congruent in suggesting the existence of an uncertain number of cryptic species within *Lu. longipalpis* s.l. in Brazil. Herein, a 525 bp-fragment of the *period* gene was used for assessing the genetic structure and phylogenetic relationship of *Lu. longipalpis* s.l. populations in Brazil. We performed two set of analyses, first we originally sequenced three populations (Passira, Santarém and Teresina) of *Lu. longipalpis* s.l. and compared them. Thereafter, we performed a global analysis including in our dataset other three pairs of sympatric populations of *Lu. longipalpis* s.l. from three Brazilian localities available in GenBank. Fixed single nucleotide polymorphisms (SNPs) sharing, maximum likelihood inference, genetic structure and haplotype analyses revealed the presence of two genetic groups, one composed of Teresina population, and the other encompassing Passira and Santarém populations. The global analysis reflected the first of its kind, and two prominent groups were observed: the clade I comprising Teresina 1S, Bodocó 1S, Caririaçu 1S and Sobral 1S; and the clade II encompassing Passira 2S, Santarém 1S, Bodocó 2S, Caririaçu 2S and Sobral 2S. Genetic differentiation data suggested a limited gene flow between populations of the clade I *versus* clade II. Our results disclosed the presence of two prominent genetic groups, which could reasonably represent populations of *Lu. longipalpis* s.l. whose males produce the same courtship song.

1. Introduction

Lutzomyia longipalpis (Lutz and Neiva, 1912) (Diptera: Psychodidae) is a hematophagous dipteran with a catholic feeding behaviour and great medical and veterinary importance (Sousa-Paula et al., 2020)(). This phlebotomine sand fly is notable for being anthropophilic and well-adapted to anthropic environments (Deane and Deane, 1957; Salomón et al., 2015). It plays a pivotal role in the transmission of *Leishmania infantum*, the causative agent of visceral leishmaniasis (VL) in the New World (Serafim et al., 2020). *Lutzomyia longipalpis* has a wide and discontinuous geographical distribution in the Neotropics, reported from Mexico and in full expansion to the north of Argentina and Uruguay (Salomón et al., 2011; (Sousa-Paula et al., 2020)). In Brazil, the country recording more than 95% of human VL cases in the continent (Pan American Health Organization, 2019), *Lu. longipalpis* is widespread in 24 out of 27 states (Galati, 2018; Sousa-Paula et al., 2020).

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Lutzomyia longipalpis was firstly described as *Phlebotomus longipalpis* based on a single male and few female specimens collected in the south-eastern Brazil (Lutz and Neiva, 1912). Posteriorly, it was replaced into the genus *Lutzomyia* (França, 1924) and no taxonomic appraisal was performed since then (Galati, 2018). Interestingly, in the late 1960s, phenotypic variations were observed between males from different Brazilian populations (Mangabeira Filho, 1969). It was noted that different male populations can present either one pair of spots on third abdominal tergites or two pairs on third and fourth ones, namely 1S (from 'one spot') and 2S (from 'two spots') phenotypes, respectively (Ward et al., 1983). Although unclear at that time if these phenotypes were intra- or interspecific variations, this was the first evidence that different taxa could be hidden under the *Lu. longipalpis* nominal species. Since then, there has been growing interest in the taxonomic status of *Lu. longipalpis* (Souza et al., 2017).

Currently, it is acknowledged that *Lu. longipalpis* and other closely-related species (i.e., *Lutzomyia alencari, Lutzomyia cruzi, Lutzomyia gaminarai, Lutzomyia matiasi* and *Lutzomyia pseudolongipalpis*) comprise a species complex, the so-called *Lu. longipalpis* complex (Sousa-Paula et al., 2021). In addition, there is solid evidence underpinning the existence of an uncertain number of cryptic species under the name *Lu. longipalpis* (hereafter also referred as *Lu. longipalpis* sensu lato) in Brazil (Bauzer et al., 2007; Souza et al., 2017).

By definition, cryptic species are identical in morphological grounds, but can be differentiated by molecular approaches (Bickford et al., 2007; Struck et al., 2018; Sousa-Paula et al., 2021). In cryptic speciation, the selection pressure may act less on morphology rather than other traits, such as physiology and reproduction (Struck et al., 2018), which may explain the absence of diagnosable morphological traits between populations of *Lu. longipalpis* s.l. Nevertheless, populations of *Lu. longipalpis* s.l. have been successfully assessed through molecular analyses, such as phylogenetic inference and genetic structuring (Bauzer et al., 2002a, 2002b; Araki et al., 2009, 2013; Lima Costa et al., 2015; Pech-May et al., 2018).

Nuclear genetic markers have been applied for assessing different populations of *Lu. longipalpis* s.l. In particular, genes associated to the circadian rhythm and organism fitness have been useful markers, such as the *period* gene (Bauzer et al., 2002b; Mazzoni et al., 2002; Bottecchia et al., 2004; Araki et al., 2009; Salomón et al., 2010; Lima Costa et al., 2015). The *period* gene was firstly studied in fruit flies (Drosophilidae) (Gleason, 2005). Mutations in this gene are associated to different types of songs produced by male flies that flap their wings during the courtship, which in turn can vary between *Drosophila*-closely related species (Gleason, 2005). Moreover, it is acknowledged that those courtship song variations are liable for reproductive isolation between sibling species [i.e., individuals that are morphologically exceedingly similar, if not identical, but are reproductively isolated (Mayr, 1963)] and, consequently, they play an important role in the speciation process of this group (Marie-Orleach et al., 2019).

Interestingly, phlebotomine sand flies exhibit similar sexual behaviour (Bray and Hamilton, 2007). Moreover, different populations of *Lu. longipalpis* s.l. can produce different types of courtship songs as well (Vigoder et al., 2015). In Brazil, two main types of songs are produced by *Lu. longipalpis* s.l. males: the Burst-type song, which has a homogeneous composition between different populations singing it; and a more variable, the Pulse-type song, with at least five variations in its composition (Vigoder et al., 2015). More recently, it has been suggested that the type of courtship song may be determinant for insemination success of *Lu. longipalpis* s.l. females (Vigoder et al., 2020). Courtship songs are therefore potentially an important piece for understanding the speciation process of *Lu. longipalpis* s.l. In the same way, nucleotide variations in the *period* gene have been congruent in depicting the different types of songs produced by *Lu. longipalpis* s.l. and it appears as reliable marker for evolutive studies in this group (Araki et al., 2013). Indeed, analyses of single nucleotide polymorphisms (SNPs) fixed between populations of *Lu. longipalpis* s.l. have been pointed out as possible markers for diagnosing the cryptic species of *Lu. longipalpis* s.l. (Lima Costa et al., 2015).

Regarding groups of cryptic species, population genetic studies are particularly important because can provide information about their taxonomic status, geographical distribution, as well as to estimate the gene flow between populations (McCoy, 2008). Expanding our knowledge on the genetic structure and diversity for a large number of *Lu. longipalpis* s.l. populations is essential to understand its evolutionary patterns and to map cryptic species distribution (Araki et al., 2009; Lima Costa et al., 2015). In the current study, we sequenced and analysed a fragment of the *period* gene for assessing the population structure and phylogenetic relationship of three allopatric populations of *Lu. longipalpis* s.l. from Brazil. Additionally, we carried out a global analysis comparing our populations with others previously studied.

2. Material and methods

2.1. Phlebotomine sand fly populations

Lutzomyia longipalpis s.l. samples analysed herein came from three colonies maintained at Aggeu Magalhães Institute (Fiocruz Pernambuco) in Recife, Brazil. These colonies originated from three Brazilian municipalities, namely Passira (Pernambuco state) (7°58′37.7" S, 35°35′34" W), Teresina (Piauí state) (5°05′36.2" S, 42°52′52.2" W), and Santarém (Pará state) (2°27′05.4" S, 54°46′21.9" W) (Fig. 1).

Both Passira and Teresina colonies were established from wild specimens collected over May and July 2019, respectively. The individuals from both Passira and Teresina included in the present study were from both F1 and F2 generations. The Santarém colony was established from specimens originated from a colony maintained since 2012 at Leônidas and Maria Deane Institute (Fiocruz Amazônia) in Manaus, Brazil. The individuals from Santarém included in the present study were from >F80 generation. The colonies were maintained based on protocols by Volf and Volfova (2011) and Lawyer et al. (2017) with adaptations, as follows: immatures stages were reared in plastic pots with a thin layer (~1 cm) of white plaster of Paris and stored at 26 ± 1 °C and 80% relative humidity (RH) in plastic boxes with a layer of sand, which was slightly wet, on the bottom. The larvae were fed with a mix of rabbit and hamster faeces and rabbit and hamster chow in a proportion of 1:1. Adults were kept in fabric-net cages (20 cm^3) at 25 ± 1 °C and >

Fig. 1. Localities where populations of *Lutzomyia longipalpis* sensu lato were collected for laboratory colonization.

80% RH without a light cycle. Apple slices were provided as sugar source *ad libitum*. Females were able to take a blood-meal on anaesthetized hamsters (Institutional Animal Care and Use Committee's license of the Fiocruz Pernambuco: 147/2019).

Based on examinations of both wild and F1 individuals, males from Passira display the 2S phenotype, whereas those from Santarém and Teresina belong to the 1S phenotype.

2.2. DNA extraction

Male and female phlebotomine sand flies from each population were placed individually into 1.5 ml microcentrifuge tubes. Genomic DNA extraction was performed using Chelex®100 resin (BioRad, Berkeley, California, USA), as described elsewhere (Lima Costa et al., 2015). Then, the extracted DNA was used as template in PCR amplifications.

2.3. PCR amplification and sequencing

Conventional PCR amplifications were carried out using the primers 5'–AGCATCCTTTTGTAGCAAAC–3' (5Llper2, forward) and 5'–TCAGATGAACTCTTGCTGTC–3' (3Llper2, reverse), which amplify a fragment (Fig. 2a) of ~500 bp of the *period* gene of phlebotomine sand flies (Mazzoni et al., 2002). The final reaction volume of 25 µl contained 7.5 µl of DNA-free water, 12.5 µl of GoTaq® Colorless Master Mix (Promega, Madison, WI, USA), 1.5 µl of each primer at a concentration of 10 pmol/µl and 2 µl of template DNA. The thermal cycling conditions were as follows: initial denaturation at 95 °C for 5 min; 30 cycles of 95 °C/30 s, 50 °C/30 s, and 72 °C/1 min; and a final extension at 72 °C/10 min. Aliquots of 2 µl from each PCR product were analysed by electrophoresis in a 0.5% agarose gel stained with ethidium bromide (stock concentration 10 mg/ml) and visualized under a UV trans-illuminator. Then, the remaining PCR products were purified using PureLink™ PCR Micro Kit (Invitrogen, USA), following the manufacturer's recommendations.

The purified PCR products were prepared using BigDye™ Terminator v3.1 Matrix Standard Kit (Applied Biosystems, Foster City, CA). Bi-directional sequencing reactions were carried out in a 3500xL Genetic Analyzer (Applied Biosystems, Foster City) using the same primers as for PCR.

2.4. Phylogenetic and genetic structure analyses

For each individual phlebotomine sand fly, from two to eight sequences were generated. These sequences were analysed with the Staden Package v.2.0.0b11 (Staden et al., 2000), which was used for consensus assembly based on the Phred quality score ≥30. Heterozygous individuals (i.e., individuals with at least one heterozygous site represented by IUPAC nucleotide ambiguity codes) were phased in DnaSP

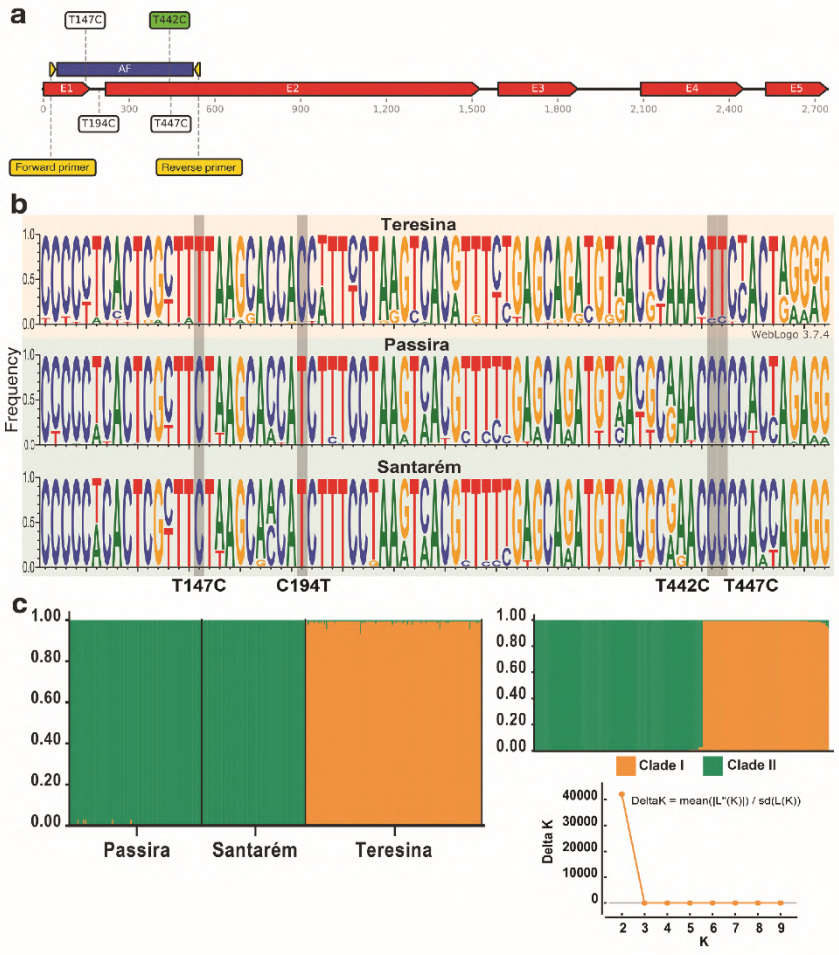

Fig. 2. Representation of the *period* gene, parsimony informative sites and genetic structure assessment of three allopatric populations of *Lutzomyia longipalpis* sensu lato. (a) The analysed fragment (AF) in the present study is represented in blue. The exons (E) are represented by red boxes. SNPs are represented by white and green (novel fixed SNP) boxes. (b) Parsimony informative sites in a 525 bp-fragment of the *period* gene. Fixed SNPs between populations are highlighted by grey boxes. (c) Genetic structure assessed by STRUCTURE software and Delta K method disclosing two genetic groups. The individuals are sorted by population (left) and genetic groups (right). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

v.6.12 (Rozas et al., 2017) using the algorithm provided by PHASE (Stephens and Donnelly, 2003) with the default parameters. This approach uses a coalescent-based Bayesian method for haplotype reconstruction. For these samples, two sequences (haplotype A and B) were included in downstream analyses. The high-quality consensus sequences were deposited in GenBank under the accession numbers: MW879754–MW879868, MW879869–MW879958 and MW879959–MW880111.

Sequence alignments were performed using ClustalW (Larkin et al., 2007) and MUSCLE algorithms (Edgar et al., 2004) implemented in MEGA7 v.7.0.26 (Kumar et al., 2016). Nucleotide polymorphisms were assessed by analyses of parsimony informative sites using MEGA7. Then, the WebLogo3 v.3.7 (Crooks et al., 2004) was used for polymorphism frequencies viewing. The positions of the polymorphisms were sorted considering the VectorBase (https://vectorbase.org) annotation for the *period* gene (accession number: LLOJ000134-RA; version LlonJ1.5), i.e., considering the first nucleotide of the exon 1.

Phylogenetic inferences were performed based on maximum-likelihood (ML) reconstruction method using IQ-TREE v.2 with 100,000 replicates of the bootstrap ultrafast method (Minh et al., 2020). The best-fit model of nucleotide substitution was calculated using ModelFinder implemented in IQ-TREE (Kalyaanamoorthy et al., 2017) and chosen according to Bayesian information criterion (BIC). Additionally, we also use a shorter fragment (266 bp) of the *period* gene of *Lutzomyia cruzi* (GenBank access number: HM059888) as outgroup. The ML trees were visualised and edited using the tool iTOL v.4 (Letunic and Bork, 2019).

The population structure was inferred using STRUCTURE v.2.3.4 (Pritchard et al., 2000) based on Bayesian clustering approach, which assign individuals into genetic clusters (K). In order to estimate the number of genetic clusters, 10 independent interactions were performed for each K value (1–10). Markov chain Monte Carlo (MCMC) simulations were performed with 1,000,000 steps with 10% of burn-in period. Posteriorly, analyses of ΔK were performed to determine the value of K best-fits the data (Earl and vonHoldt, 2012).

The pair-wise fixation index (F_{ST}) (based on 1,000 simulations), number of migrants per generation (Nm), and Tajima's D neutrality test were calculated using Arlequin v.3.5.2 (Excoffier and Lischer, 2010). The nucleotide diversity (π), average number of substitutions per site among populations (Dxy), total number of substitutions per site among populations (Da), number of shared polymorphisms among populations (Ss), number of fixed differences among populations (Sf), number of haplotypes and haplotype diversity (Hd) were calculated using DnaSP. Haplotype networks were inferred using a median-joining algorithm (Bandelt et al., 1999) implemented in PopArt software (http://popart.otago.ac.nz/).

In general, we carried out two set of analyses: firstly, the sequences of the three populations originally generated in the present study were analysed. Thereafter, we performed a global analysis including into the same dataset populations of *Lu. longipalpis* s.l. previously published by Lima Costa et al. (2015) (GenBank accession numbers: 808791686 and 566335000), which comprise 1S and 2S sympatric populations of *Lu. longipalpis* s.l. from three Brazilian localities, namely Bodocó (Pernambuco state), Caririaçu and Sobral (Ceará state). We have chosen these datasets because they comprise the same fragment of the *period* gene sequenced in the present study.

3. Results

A 525 bp fragment of the *period* gene was successfully sequenced from 226 colonized individuals of *Lu. longipalpis* s.l. Of these, 75 individuals were from Santarém (35 females and 40 males), 75 from Passira (41 females and 34 males), and 76 from Teresina (39 females and 37 males) (Table 1).

Polymorphism analyses resulted in 14.7% (77/525) of parsimony informative sites between the populations of Passira, Santarém and Teresina. Of these, four fixed SNPs were observed: T147C, C194T, T442C and T447C. The populations from Passira 2S and Santarém 1S shared the second allele for all fixed SNPs, whereas for Teresina 1S the first allele is fixed for C194T and most frequent in the remaining three SPNs. All these SNPs can clearly be employed to depict the populations from Teresina to Passira and Santarém (Fig. 2b).

The ML tree revealed the presence of two separated and strongly supported clades (node support = 99) (Fig. 3). Individuals from Teresina (clade I) comprised a distinct clade when compared to those from Passira and Santarém (clade II). Remarkably, the clade II appears as a derived group from clade I. This topology is also observed when a shorter fragment of *Lu. cruzi* was used as outgroup (Supplementary material 1: Fig.S1a). Additionally, genetic structure analyses underpinned the presence of two genetically separated groups ($K = 2$). This finding is congruent to ML analysis and fixed SNPs, i.e., the populations from Passira and Santarém comprise a different genetic group when compared to Teresina (Fig. 2c).

As far as interpopulation genetic differentiation, Teresina pairwise F_{ST} values were significantly highest when compared with Passira ($F_{ST} = 0.422$, $P < 0.0001$) and Santarém ($F_{ST} = 0.498$, $P < 0.0001$). In contrast, Passira and Santarém showed a low divergence degree between them ($F_{ST} = 0.129$, $P < 0.0001$) (Table 2). Regarding number of migrants per generation, the population from Teresina showed a low value compared with both Passira ($Nm = 0.684$) and Santarém ($Nm = 0.504$) (Table 2). Otherwise, Passira and Santarém showed a higher value ($Nm = 3.364$). Altogether, these data support Teresina as a genetic group separated of Passira and Santarém, suggesting a limited gene flow between these two groups.

A total of 135 haplotypes was detected across the three populations (see Supplementary Material 2: Dataset). The haplotypes H_2 and H_19 ($n = 13$ sequences), H_61 ($n = 28$) and H_104 ($n = 12$) were the most frequent in Teresina, Passira and Santarém individuals, respectively. Although there was no haplotype sharing between Passira and Santarém, these populations comprised the same haplogroup, differing from Teresina (Fig. 4). The absence of haplotype sharing between the populations from Passira and Santarém may be associated to the high values of haplotype diversity found in the present study (Table 1).

3.1. Global analyses

For this analysis, 171 sequences from three pairs of sympatric populations of *Lu. longipalpis* s.l. from three localities available in GenBank were added to our dataset. Polymorphism analysis revealed the presence of 82 (out of 525; 15.6%) parsimony informative sites. Of these, the

Table 1
Summary of genetic diversity between populations of *Lutzomyia longipalpis* sensu lato and neutrality test.

Population	*n*	S	Tajima's D	Hd	π ± Sd	NS	h	k
Passira S	75	120	0.40086	0.97227	0. 01545 ± 0.00036	38	60	8.020
Santarém	75	90	1.51767	0.82572	0. 01097 ± 0.00066	19	18	5.698
Teresina	76	151	0.61550	0.96389	0. 02080 ± 0.00049	50	57	10.748
Total	226	361					135	

Abbreviations: n, number of individuals; S, number of DNA sequences (see Methods for details); Tajima's D, Tajima test of neutrality; Hd, haplotypic diversity; π ± Sd, nucleotide diversity and standard deviation; NS, number of polymorphic sites; h, number of haplotypes; k, average number of nucleotide differences.

Fig. 3. Maximum likelihood tree (TPM3+F+I+G4 model) using a 525 bp-fragment of the *period* gene from three allopatric populations of *Lutzomyia longipalpis* sensu lato. Bootstrap values above to 60% are represented in the nodes. Some low-bootstrap nodes were collapsed for best tree visualization. Population from Teresina 1S comprises a basal group (clade I; orange) compared to populations from Passira 2S and Santarém 1S, which appears as a derived group (clade II; green). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Table 2
Genetic differentiation between three allopatric populations of *Lutzomyia longipalpis* sensu lato in Brazil.

Populations		F_{ST}	Nm	Dxy	Da	Ss	Sf
Passira	Santarém	0.129*	3.364	0.01506	0.00200	17	0
Santarém	Teresina	0.498*	0.504	0.03244	0.01678	5	1
Teresina	Passira	0.422*	0.684	0.03116	0.01329	11	1

Abbreviations: F_{ST}, pair-wise genetic differentiation; Nm, number of migrants per generation; Dxy, average number of nucleotide substitutions per site between populations; Da, number of net nucleotide substitutions per site between populations; Ss, number of shared polymorphisms between pairs of population; Sf, number of fixed differences between pairs of populations. Significance of F_{ST} values was evaluated by 1,000 random permutations (* $P < 0.001$).

above-mentioned fixed SNPs were also detected and can be useful for distinguishing both allopatric and sympatric populations of *Lu. longipalpis* s.l. analysed herein (Supplementary Material 3: Fig.S2).

In a similar way to the initial analyses, the ML analyses revealed the presence of two prominent groups: the clade I comprising the populations from Teresina 1S, Bodocó 1S, Caririaçu 1S and Sobral 1S; and the clade II comprising Passira 2S, Santarém 1S, Bodocó 2S, Caririaçu 2S and Sobral 2S (Supplementary Materials 1 and 4: Figs. S1b and S3a). The separation of these two clades was well supported (node support = 95), with the clade II as a group deriving from clade I. Additionally, this assemblage was underpinned by genetic structure analyses, which indicated the existence of two ($K = 2$) genetically separated populations (Supplementary Material 4: Fig.S3b).

The data of genetic differentiation were congruent with the above findings (see Supplementary Material 2: Dataset). Moderate and significant pairwise F_{ST} values were observed between populations within clades I and II (0.422–0.665). In contrast, comparisons between populations within the same clade showed lower genetic divergence (F_{ST} = 0.0–0.173). As far as the number of migrants per generation, again, populations of the clade I compared to the clade II showed a high degree of divergence (Nm = 0.252–0.684), whereas populations of the same clade showed an elevated number of migrants, ranging from 2.388 up to extremely high values resulting in infinite migrants per generation.

Finally, the global haplotype analysis resulted in a total of 283 haplotypes across the nine populations (see Supplementary material 2: Dataset). Populations from Teresina 1S, Bodocó 1S, Caririaçu 1S and Sobral 1S composed a distinct haplogroup than Passira 2S, Santarém 1S, Bodocó 2S, Caririaçu 2S and Sobral 2S, in agreement with the above results (Supplementary material 5: Fig. S4). Remarkably, Passira 2S and Caririaçu 2S shared the haplotype Hap_82, as well as Caririaçu 1S and Sobral 1S (Hap_203), Bodocó 2S and Sobral 2S (Hap_172), and Caririaçu 2S and Sobral 2S (Hap_209 and Hap_210).

4. Discussion

As previously mentioned, studies have detected mutations in the

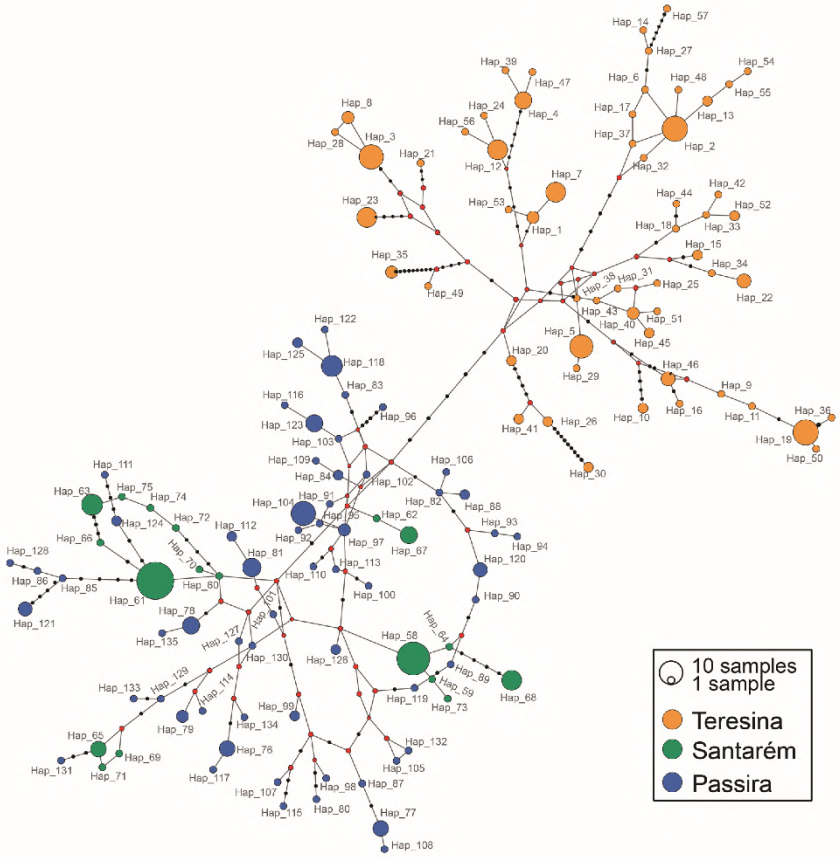

Fig. 4. Median-joining haplotype network of three populations of *Lutzomyia longipalpis* sensu lato based on a 525 bp-fragment of the *period* gene. Haplotype frequency is represented by the sizes of the circles. Missing haplotypes are represented in red, and the mutational steps are represented by black circles.

period gene of *Lu. longipalpis* s.l. and these have been useful as molecular markers to distinguish populations across Brazil (Souza et al., 2017). Fixed SNPs were suggested by Lima Costa et al. (2015) for distinguishing sympatric populations of *Lu. longipalpis* s.l. The authors provided a set of four fixed SNPs, of which two could be reliable for identification of the cryptic species of *Lu. longipalpis* s.l. In the present study, we detected three of these fixed SNPs (T147C, C194T, and T447C) and an additional one (T442C). Regarding Passira, Santarém and Teresina populations, these four SNPs clearly can be used for distinguishing the clades encompassing Teresina and Passira/Santarém. Additionally, in the global analysis including the other six populations, it could be noted that no allele is exclusive of a clade. For instance, for the SNPs T147C, T442C and T447C, the allele "C" is fixed for individuals of the clade II, whereas the second allele is highly frequent in individuals of the clade I, but few individuals presented an allele "C" as well. In contrast, for the SNP C194T, the allele "C" is the only one present in individuals of the clade I, whereas the allele "T" is the most frequent for individuals of the clade II, but, again, few individuals presented an allele "C" as well. These results support the discriminatory ability of this SNP set for depicting the populations of *Lu. longipalpis* s.l. Nonetheless, further studies involving a larger number of populations would better elucidate if these low-frequency alleles are rare allele or a sequencing artefact, or even results of introgressive hybridization.

Previous studies analysed the courtship song produced by male populations from Sobral (1S and 2S), Teresina 1S (Araki et al., 2009) and Passira (phenotype not mentioned; Vigoder et al., 2015). While Sobral 1S and Teresina 1S populations produce a Pulse-type song, Passira and Sobral 2S populations produce a Burst-type song. In addition, other populations from Pará state (i.e., Ilha do Marajó 1S, Camará, Barcarena, and Cametá) produce a Burst-type song as well (Vigoder et al., 2015). In this regard, it is plausible to suppose that the Santarém 1S population may sing the same Burst-type song in agreement with other Amazonian populations from Pará state. Accordingly, our analyses of ML inference, genetic structure and haplotype network support the presence of two prominent groups: the clade I (composed by Teresina 1S, Bodocó 1S, Caririaçu 1S and Sobral 1S) and the clade II (composed by Passira 2S, Santarém 1S, Bodocó 2S, Caririaçu 2S and Sobral 2S). As such, the clades observed in the present study may reasonably reflect the type of courtship produced by each population, as previously reported (Bauzer et al., 2002a, 2002b; Souza et al., 2002). This is not an unexpected result, since the *period* gene is believed to be associated to the rhythm control of the courtship songs in phlebotomine sand flies, as occurs in *Drosophila* flies (Oliveira et al., 2001; Peixoto et al., 2001). Furthermore, the fact of populations with different abdominal-spot phenotypes grouping in the same clade (e.g., Passira 2S and Santarém 1S) reinforces that these characters may be not reliable for differentiating allopatric populations of *Lu. longipalpis* s.l. (Phillips et al., 1986; Ward et al., 1988), although they may be useful when the populations occur in sympatry (Araki et al., 2009; Lima Costa et al., 2015; Vigoder et al, 2015).

More recently, chemoreceptor genes were used for phylogenetic and genetic structure analyses comparing four populations of *Lu. longipalpis* s.l. in Brazil (Hickner et al., 2020). Remarkably, the results showed two

prominent groups, one comprising Burst-type song populations and another grouping Pulse-type song populations (Hickner et al., 2020). This finding support that the courtship song is a reliable trait for sorting the cryptic species of *Lu. longipalpis* s.l. In view of this, the *period* gene figures out as a reliable marker for depicting the cryptic species of *Lu. longipalpis* s.l. in Brazil.

It is interesting to note that the ML analyses showed the clade I as a basal group and the clade II as a derived group, as reported by Lima Costa et al. (2015). The congruence of these two studies with different populations may underpin the hypothesis of a derived group. Herein, we also used a shorter fragment of *Lu. cruzi* as outgroup and the same topology was observed, which adds weight to this hypothesis. In addition, the possibility of the populations that produce Pulse-type song being a basal group may be associated to the variations found in its song composition (Vigoder et al., 2015), i.e., a group more ancient had been more time for fixing the mutations and consequently generating more genetic diversity. Incidentally, events of secondary contact and introgressive hybridization have been pointed out for driving the genetic diversity between the populations of *Lu. longipalpis* s.l. in Brazil (Coutinho-Abreu et al., 2008; Araki et al., 2013). Further studies including more species of the *Lu. longipalpis* complex might shed light on the evolutionary history of this group.

Genetic differentiation analyses indicated a high degree of divergence between the populations belonging to clade I *versus* clade II. These values are similar to those found between *Lu. longipalpis* s.l. and *Lu. cruzi* (using a shorter 266 bp-fragment of the *period* gene) (Vigoder et al., 2010). For some populations, the F_{ST} values (e.g., Santarém 1S *versus* Bodocó 1S = 0.641) is nearly close to values reported for cryptic species of the phlebotomine sand fly *Nyssomyia umbratilis* also based on the *period* gene (de Souza Freitas et al., 2016). Altogether, these results suggest a limited gene flow between populations within clades I and II. Crossbreeding analyses between populations of *Lu. longipalpis* s.l. have been conducted for a few Brazilian populations so far (Ward et al., 1983; Souza et al., 2008). Some populations of *Lu. longipalpis* s.l. may be reproductively isolated, especially when they occur in sympatry. As a matter of fact, crossbreeding experiments comparing a larger number of populations are advocated to clarify the reproductive barriers that may exist between populations of *Lu. longipalpis* s.l., which could generate useful data for an integrative taxonomy approach (Padial et al., 2010; Dantas-Torres, 2018).

Previous studies have suggested that some populations of *Lu. longipalpis* s.l. may better transmit *L. infantum* than others (Casanova et al., 2006, 2015). Importantly, the failure of VL control strategies in endemic countries such as Brazil may be partly explained by the limited financial resources (Dantas-Torres et al., 2019; Sousa-Paula et al., 2019). In this regarding, clarifying if different populations of *Lu. longipalpis* s.l. may act differently in the VL transmission cycle can optimize the resources available for controlling this disease. In this perspective, the ability to distinguish *Lu. longipalpis* s.l. females based on polymorphisms of the *period* gene, as previously suggested (de Souza Freitas et al., 2018), may allow further studies on the vector role of these cryptic species.

The taxonomic status of *Lu. longipalpis* s.l. has received increasing attention in recent years (Souza et al., 2017), particularly due to its pivotal role in the *L. infantum* transmission in the Americas. Currently, the existence of several cryptic species of *Lu. longipalpis* s.l. has been demonstrated by several studies (Oliveira et al., 2001; Bauzer et al., 2002a, 2002b; Araki et al., 2009, 2013; Lima Costa et al., 2015; Pech-May et al., 2018). Nonetheless, there is no consensus or formal descriptions for these cryptic species (Brandão-Filho et al., 2009; Sousa-Paula et al., 2021; Souza et al., 2017), perhaps also because the type specimen of *Lu. longipalpis* sensu stricto is currently unavailable (Brandão-Filho et al., 2009; Sousa-Paula et al., 2021). Understanding the genetic structure and phylogenetic relationship of as many populations as possible is an important step to clarify the distribution and, consequently, the formal recognition of the cryptic species of *Lu. longipalpis* s.l.

Our results disclose that the three populations of *Lu. longipalpis* s.l. originally analysed herein comprise two different genetic groups: one encompassing the population from Teresina, whereas the second one is composed of the population from Passira and Santarém. The continuous effort for studying a larger number of populations of *Lu. longipalpis* s.l. and closely related species is advocated for elucidating the evolutionary history of this species complex. Furthermore, undertaking crossbreeding studies between these populations could shed light on their biological compatibility under laboratory conditions.

Understanding the inter- and intrapopulation genetic diversity of *Lu. longipalpis* s.l. is essential to improve our knowledge of the distribution of cryptic species belonging to this species complex in Brazil and elsewhere in the Americas. Whether these cryptic species may play different roles in *L. infantum* transmission is an open question that requires further research.

CRediT authorship contribution statement

Lucas Christian de Sousa-Paula: Conceptualization, Investigation, Formal analysis, Visualization, Writing – original draft. **Lidiane Gomes da Silva:** Formal analysis, Writing – review & editing. **Wilson José da Silva Junior:** Formal analysis, Writing – review & editing. **Carlos Alberto Santiago Figueirêdo Júnior:** Formal analysis, Writing – review & editing. **Carlos Henrique Nery Costa:** Resources, Writing – review & editing. **Felipe Arley Costa Pessoa:** Resources, Writing – review & editing. **Filipe Dantas-Torres:** Conceptualization, Project administration, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2021.106031.

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4.4 Artigo 4 – Who is *Lutzomyia longipalpis* (Lutz & Neiva, 1912)?

Lutzomyia longipalpis sensu stricto foi originalmente descrita em 1912. O status taxonômico desta espécie está sob discussão há pelo menos meio século. Embora um robusto arcabouço de estudos suporte a existência de várias espécies crípticas sob o nome *L*. *longipalpis*, uma pergunta tem recebido pouca atenção: quem é a espécie orginalmente descrita por Adolpho Lutz e Artur Neiva? Essa é uma pergunta central para que as novas espécies crípticas possam ser eventualmente nomeadas. Nesse artigo nós discutimos pontos importantes que devem ser considerados para uma completa caracterização e designação de um neótipo para *L*. *longipalpis* s.s.

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Who is *Lutzomyia longipalpis* (Lutz & Neiva, 1912)?

Dear Editor,

Lutzomyia longipalpis (Lutz and Neiva, 1912) is one of the most important species of phlebotomine sand flies in the Americas due to its role as the main vector of *Leishmania infantum*, the causative agent of visceral leishmaniasis in the region (Sousa-Paula et al., 2020). Beyond its medico-veterinary relevance, the taxonomic status of *L. longipalpis* has been an issue of debate for more than a half-century. In the late 1960s, Mangabeira (1969) documented what later became the first evidence that different taxa could be hidden under the name *L. longipalpis*. Male populations of *L. longipalpis* can present slight phenotypical variations, that is, one pair of pale whitish spots on the third abdominal tergite or two pairs on the third and fourth tergites, namely 1S (from 'one spot') and 2S (from 'two spots') phenotypes, respectively (Ward et al., 1983). Nowadays, it is acknowledged that these phenotypic variations are not representative of the evolutionary lineages of *L. longipalpis* sensu lato (Sousa-Paula et al., 2021a). In fact, along the past decades, numerous behavioural, biochemical and genetic studies shed light on the existence of cryptic species, which have been collectively called *L. longipalpis* s.l. (Souza et al., 2017). The existence of reproductive barriers between these populations has been discussed (Ward et al., 1983; Souza et al., 2008). For instance, *L. longipalpis* s.l. males in Brazil can produce at least five variations in pheromone chemotypes (Spiegel et al., 2016; Palframan et al., 2018) and two main types of courtship songs (the Burst-type and Pulse-type, with the later displaying at least five quantifiable variations) (Vigoder et al., 2015). Furthermore, nuclear and mitochondrial gene markers are able to separate different populations (Souza et al., 2017). Nonetheless, these data may oftentimes point in different directions, which further complicates the matter.

Recently, Gutierrez et al. (2021) used mitochondrial DNA barcoding for analysing populations of *L. longipalpis* s.l. from different South and Central American countries. Their findings were in line with previous studies (Uribe, 1999; Bauzer et al., 2007; Souza et al., 2017; Sousa-Paula et al., 2021b) and suggested the existence of at least eight molecular operational taxonomic units (MOTUs), which may represent different species. The study was well conducted and the results are important to the discussion on the biosystematics status of *L. longipalpis* s.l. Nonetheless, we would like to discuss a key issue that did not receive the attention it deserves: who is the real *L. longipalpis* sensu stricto?

>The original description of *L. longipalpis* s.s. was based on only external characters from a single male and an uncertain number of female specimens (Lutz and Neiva, 1912). The type locality was indirectly referred as 'Ouro Fino farm' near Benjamim Constant, Além Paraíba municipality (Minas Gerais, Brazil), for the male specimen, and 'Bosque da Saúde' near São Paulo city (São Paulo, Brazil), possibly for the females (Lutz and Neiva, 1912). Unfortunately, the type specimen is unavailable. Gutierrez et al. (2021) highlighted that their results showed slight agreement with the four clades (Species A–D) reported by (Arrivillaga et al., 2003), who reported one of these clades (i.e., Species A) to be the actual *L. longipalpis* s.s. However, we now know that the so-called clade "Species A" reported by (Arrivillaga et al., 2003) includes Brazilian populations (e.g., Jacobina, Sobral and Santarém) that may differ between themselves in courtship song, aggregation-sex pheromone, and/or genetic data, possibly representing different members within the complex (for a review, see Souza et al. 2017). Therefore, we argue that the clade "Species A" could not represent a single taxon. So, the question remains.

Nevertheless, there is a pivotal study that may shed light on this question. A 1-year study conducted in Além Paraíba, near the type locality, resulted in the collection of four specimens (one female and three males) of *L. longipalpis* s.l. (Brazil et al., 2006). Apparently, voucher specimens are deposited in the collection of Centro de Referência Nacional e Internacional para Flebotomíneos (CRNIF) of the René Rachou Institute (Fiocruz Minas), Belo Horizonte, Minas Gerais, Brazil (de Castilho Sanguinette et al., 2013). Unfortunately, to the best of our knowledge, no molecular data have been generated for these or other specimens collected in Além Paraíba, which may well represent the actual *L. longipalpis* s.s. described by Lutz and Neiva (1912). Ideally, a coordinated effort to collect more samples in different areas of Além Paraíba, possibly in the region known as Benjamim Constant, is required to fix the neotype. Importantly, this taxonomic act (neotype designation) should include all available information (e.g., courtship songs, aggregation-sex pheromones, mitochondrial and nuclear markers, and morphological traits) for a complete characterization of *L. longipalpis* s.s. In this regard, it is also crucial to ensure that in the type locality there is a single population, since the existence of cryptic species occurring in sympatry has been demonstrated in some areas (Araki et al., 2009; Lima Costa et al., 2015). Finally, numerous voucher specimens and perhaps the establishment of a laboratory colony would be an essential step for paving the downstream experiments, including a complete reference genome sequencing.

Historically, the systematic status of several arthropod vectors has been an issue of debate (Sousa-Paula et al., 2021b) and in a couple of cases the designation of a neotype has been a fundamental step for solving these taxonomic conundrums. The Code of International Commission on Zoological Nomenclature (ICZN) issues that "*when no name-bearing type specimen (i.e., holotype, lectotype, syntype or prior neotype) is believed to be extant and an author considers that a name-bearing type is necessary to define the nominal taxon objectively*" a neotype may be fixed (ICZN, 1999). In fact, these were the cases of the mosquitoes *Culex quinquefasciatus* (Sirivanakarn and White, 1978) and *Aedes albopictus* (Huang, 1968), the phlebotomine sand flies *Nyssomyia intermedia* (Lutz and Neiva, 1912; Marcondes, 1996), *Phlebotomus annandalei* and *Phlebotomus glaucus* (*Phlebotomus argentipes* complex) (Ilango, 2010), and the tick *Rhipicephalus sanguineus* (Nava et al., 2018). Regarding *L. longipalpis* s.s., the absence of a name-bearing specimen and the

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need for a neotype designation have been highlighted previously (Brandão-Filho et al., 2009), but no designation has been made yet. Considering the several lines of evidence underpinning the existence of different taxa in the so-called *L. longipalpis* complex, efforts to fix a neotype are urgently needed. This is a fundamental step towards solving this long-standing taxonomic conundrum.

As mentioned above, integrative analyses including molecular markers, behavioural (courtship songs and crossbreeding) and biochemical (aggregation-sex pheromones) data would allow us to delimit the cryptic species of *L. longipalpis* s.l. more precisely. As a matter of fact, unless a name-bearing specimen is made available, we will not be able to ascertain which MOTU reported by Gutierrez et al. (2021) is *de facto* the actual sensu stricto species. In this sense, none of the remaining MOTUs (putative new species?) can be formally described prior to the *L. longipalpis* s.s. neotype designation. In these cases, caution should be exercised to avoid premature description of new species that may became be synonymized in the future, as emphasized elsewhere (Dantas-Torres, 2018).

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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4.5 Artigo 5 – Biological compability of allopatric populations of *Lutzomyia longipalpis* sensu lato[4]

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ABSTRACT

Lutzomyia longipalpis is the main vector of *Leishmania infantum* in the Neotropics and is a classic example of arthropod vector entangled in a taxonomic *conundrum*. Since observations that male populations can present two phenotypes (namely 1S and 2S), the taxonomic status of *L*. *longipalpis* has been under debate. Crossbreeding experiments between different allopatric and sympatric populations have pointed out the existence of reproductive isolation. Currently, production of different sex-aggregation pheromones, copulatory songs and genetic data underpin the existence of an uncertain number of cryptic species under the name *L*. *longipalpis* and that are collectively called *L*. *longipalpis* sensu lato. More recently, we have analysed the genetic structure and phylogenetic relationship of three colonized populations of *L*. *longipalpis* s.l. The data disclosed the population from Teresina (Piauí state; 1S phenotype) composing a different clade when compared to Passira (Pernambuco state2S phenotype) and Santarém (Pará state; 1S phenotypes). Furthermore, this data suggested a limited gene flow between these two clades, suggesting the existence of reproductive barriers between these populations. In this perspective, in the present study we carried out crossbreeding experiments towards evaluating the biological compatibility between *L*. *longipalpis* s.l. populations from Passira, Teresina and Santarém. Our results show no difference in terms of egg hatching

[4] Manuscrito não publicado.

rate and offspring viability from crosses between individuals of Passira and Santarém. However, crosses between Santarém and Teresina resulted an asymmetric reproductive isolation, with no eggs hatched in male Santarém *versus* female Teresina cross, and a low egg hatching rate between male Teresina and female Santarém cross. In addition, Passira and Teresina crosses resulted in an extremely low egg hatching rate in both directions. Our crossbreeding data underpin the existence of reproductive barriers between the populations from Passira and Santarém when compared to Teresina. These findings fit with genetic data previously analysed and add weight to the hypothesis of different species under the name *L*. *longipalpis*.

Key words: Crossbreeding. Cryptic species. Phlebotominae. Reproductive isolation.

Introduction

Lutzomyia longipalpis (Lutz & Neiva, 1912) is the main vector of *Leishmania infantum* in the American continent (Brazil et al., 2015; Sousa-Paula et al. 2020). In addition, it is a classic example of an important vector entangled in a taxonomic *conundrum* (Uribe 1999; Bauzer et al. 2007; Souza et al. 2017; Sousa-Paula and Dantas-Torres 2021).

Since the late 1960s, a report of slight phenotypic variations in males posed doubts on the existence of a single species under the name *L*. *longipalpis* (Mangabeira Filho 1969). Males can present two forms: one displaying a single whitish spot on the fourth abdominal tergite and another presenting two pairs of spots on the third and fourth tergites (Ward et al. 1983). These variations have since been named one-spot (1S) and two-spots (2S) phenotypes, respectively (Ward et al. 1983).

During the 1970s, *L*. *longipalpis* colonies from Brazilian populations with both male phenotypes were reared in the United Kingdom (Killick-Kendrick et al. 1973; White and Killick-Kendrick 1975). Crossed populations displayed a low fertility, which suggested a possible reproductive isolation between 1S and 2S populations. Thereafter, crossbreeding studies showed the existence of partial reproductive isolation between both allopatric and sympatric Brazilian populations of *L*. *longipalpis* (Ward et al. 1983, 1988; Souza et al. 2008).

Nonetheless, these studies showed male spot phenotypes are not directly correlated with reproductive isolation.

The sexual behaviour of *L*. *longipalpis* involves release of sex-aggregation pheromones and acoustic signs (copulatory songs), both produced by males (Killick-Kendrick 1999; Brazil and Brazil 2018). Different *L*. *longipalpis* male populations can eventually produce different types of sex-aggregation pheromones (Hamilton et al. 2005; Watts et al. 2005; Maingon et al. 2005; Brazil et al. 2009; Spiegel et al. 2016) and copulatory songs (Souza et al. 2004; Vigoder et al. 2015, 2020). Also including genetic data from mitochondrial and nuclear markers (Araki et al. 2009, 2013; Souza et al. 2017), these different lines of evidence underpinned the existence of an uncertain number of cryptic species that are collectively called *L*. *longipalpis* sensu lato (Sousa-Paula et al. 2021a). Nonetheless, these species have not yet been formally described (Brandão-Filho et al. 2009; Sousa-Paula and Dantas-Torres 2021).

Historically, taxonomy and systematics of phlebotomine sand flies have been based on a typological concept, using morphological traits as the criterion for species delineation (Galati 2018). In spite of that, integrative approaches have increasingly been used towards understanding the evolutionary history and solving taxonomic problems in this group of insects (Depaquit 2014; Bates et al. 2015). In the New World, where more than 540 phlebotomine sand fly species are present, about 30% of the valid species are morphologically indistinguishable when compared to one or more species (Sousa-Paula et al. 2021a). Incidentally, some of these species may play role as vectors of *Leishmania* parasites or other pathogen (Sousa-Paula et al. 2021a), denoting the need of new tools for delimiting and depicting them.

Recently, we performed a set of analyses approaching phylogenetic and genetic structure assessment for comparing three allopatric populations of *L*. *longipalpis* from the north and north-eastern regions from Brazil (Sousa-Paula et al. 2021b). Our findings suggested the presence of two prominent clades. The clade I encompassed the population from Teresina (Piauí state, north-eastern), whose males show the 1S phenotype, whereas the clade II included populations from Passira (Pernambuco state, north-eastern) and Santarém (Pará state, north), whose males show 2S and 1S phenotypes, respectively. Remarkably, the genetic data suggested a limited gene flow between these two clades, which

suggested the existence of reproductive barriers between these populations. In this perspective, in the present study we carried out crossbreeding experiments towards evaluating the biological compatibility between *L*. *longipalpis* s.l. populations from Passira, Teresina and Santarém.

Methods

Lutzomyia longipalpis s.l. populations

Lutzomyia longipalpis s.l. samples included in this study originated from three colonies maintained at Aggeu Magalhães Institute (Fiocruz Pernambuco) in Recife, Brazil. These colonies originated from three Brazilian municipalities, namely Passira (Pernambuco state) (7°58'37.7" S, 35°35'34" W), Teresina (Piauí state) (5°05'36.2" S, 42°52'52.2" W), and Santarém (Pará state) (2°27'05.4" S, 54°46'21.9" W).

The colonies were maintained as described elsewhere (Sousa-Paula et al. 2021b). Briefly, immatures stages were reared in plastic pots with a thin layer (~1 cm) of white plaster of Paris and stored at 26 ±1 °C and 80% relative humidity (RH) in plastic boxes with a layer of sand slightly wet on the bottom. The larvae were fed with a mix of rabbit and hamster faeces and rabbit and hamster chow in a proportion of 1:1. Adults were kept in fabric-net cages (20 cm^3) at 25 ±1 °C and >80% RH, without a light/dark cycle. Apple slices were provided as sugar source *ad libitum*. Females were allowed to take a blood-meal on anaesthetized hamsters (Institutional Animal Care and Use Committee's license of the Fiocruz Pernambuco: 147/2019).

For the crossbreeding experiments described below, we used phlebotomine sand flies from F18 from Teresina, F20 from Passira and >F80 from Santarém.

Crossbreeding experiments

Late pupae of *L*. *longipalpis* s.l. were individualised into 1.5 µl tubes and checked daily until adult emergence. Adults were sorted into single-sex cages for each population. This step was performed to ensure no copulation before the experiments. Crossbreeding experiments were carried out using 3 to 5-day-old virgin adults. During these experiments, males and females were left together for a total of 72 hours (4 days), under the above-mentioned laboratory conditions.

On day 1, groups of 15–25 virgin couples were transferred to same mating cages (15 cm³), as follows: inter-population crosses, i.e., males from Passira (♂PAS) *versus* females from Santarém (♀SAN) or Teresina (♀TER); males from Santarém (♂SAN) *versus* females from Passira (♀PAS) or ♀TER; males from Teresina (♂TER) *versus* ♀PAS or ♀SAN. Intra-population crosses (i.e., ♂PAS x ♀PAS; ♂SAN x ♀SAN; ♂TER x ♀TER) were likewise conducted as controls. On day 2, an anaesthetized hamster was placed inside the cage for female blood-feeding, for 1 hour in the dark. When necessary, we repeated this procedure after 24 h to boost the female engorgement rate. Finally, on day 4, blood-fed females were individually transferred into a glass vial containing a small strip of filter paper for oviposition. After female death, the number of eggs laid was recorded and ovaries were also dissected to observe the eventual presence of retained eggs. The final sample size (*n*) was considered the females that laid eggs before to die for each cross. Total egg production was considered as the sum of laid and retained eggs. The egg hatching rate, i.e., eggs that produced larva, was measured as number of larvae hatched from each female in relation to the number of eggs laid. Finally, the complete developmental cycle by adult emergence was followed-up.

Data analyses

The number of eggs laid, and the egg hatching were calculated along with their exact 95% confidence intervals (95% CI). The standard deviation (SD) was calculated for means obtained from the phlebotomine sand fly life cycle data. A one-way ANOVA was performed to compare the effect of crosses on production of eggs, egg hatching rate and life cycle data. The statistics analyses were performed using the Statistix 9 (https://www.statistix.com/).

Results

We carried out crosses between three allopatric populations of *L*. *longipalpis* s.l. Regarding intra-population crosses, the mean of eggs laid per female was 44.2 (95% CI: 38.3–50.1) for ♂PAS x ♀PAS, 34.4 (95% CI: 27.6–41.3) for ♂SAN x ♀SAN, and 32.6 (95% CI: 26.1–39.0) for ♂TER x ♀TER. (Figure 1) A one-way ANOVA was performed to compare the effect of crosses on egg production. There was a statistically significant difference in production of eggs from ♀PAS

compared to ♀SAN and ♀TER ($F_{(2, 43)}$ = 4.38, P = 0.019) (Table 2). As expected, all intra-population crosses resulted in viable offspring, except for a single female from Passira and another from Santarém. The egg hatching rate ranged from 54.9 to 61.9% in the three populations, with no difference between them ($F_{(2, 41)}$ = 1.57, P = 0.220). The total number of eggs, eggs hatched, egg hatching rate, as well as the mean number of eggs and the egg hatching per female are reported in Table 1.

Table 1. Crossbreeding between allopatric populations of *Lutzomyia longipalpis* s.l.

Crosses		*n* [a]	Total eggs laid	Total eggs hatched	Egg hatching rate	Mean eggs/female	Egg hatching rate/female	Male phenotype reared
Intra-population	♂PAS x ♀PAS	23	1016	629	61.9%	44.17	64.0%	2S
	♂SAN x ♀SAN	11	379	212	55.9%	34.45	51.3%	1S
	♂TER x ♀TER	10	326	179	54.9%	32.6	54.1%	1S
Inter-population	♂PAS x ♀SAN	6	216	121	56.0%	36.00	53.7%	2S
	♂SAN x ♀PAS	11	310	158	51.0%	28.18	50.0%	2S
	♂TER x ♀SAN	11	233	25	10.7%	21.18	7.3%	1S
	♂SAN x ♀TER	10	286	0	0.00%	28.60	0.0%	-
	♂PAS x ♀TER	25	643	44	6.8%	25.72	3.4%	2S
	♂TER x ♀PAS	5	107	1 [b]	0.9%	21.40	1.4%	-

Note: PAS = Passira; SAN = Santarém; TER = Teresina; ♂= male; ♀ = female.
[a] Females laid eggs before died.
[b] A single female.

Regarding inter-population crosses, mating was observed in all population combinations. The mean number of eggs varied from 21.2 to 36 per female. There was no difference in terms of egg production between inter-population crosses ($F_{(5, 62)}$ = 1.30, P = 0.275). Crosses in both directions between Passira and Santarém populations resulted in viable hybrids until their adult stage. The egg hatching rate per female was 53.67% (95% CI: 20.43– 86.87) for ♂PAS x ♀SAN, and 50.02% (95% CI: 24.44– 61.79) for ♂SAN x ♀PAS crosses. On the other hand, crosses between Santarém and Teresina populations resulted in an asymmetric reproductive isolation, i.e., only one direction of the crosses produced viable offspring. While a low egg hatching rate per female (7.3%; 95% CI: 0.0–21.7) was observed in the cross ♂TER x ♀SAN, no egg hatched in the ♂SAN x

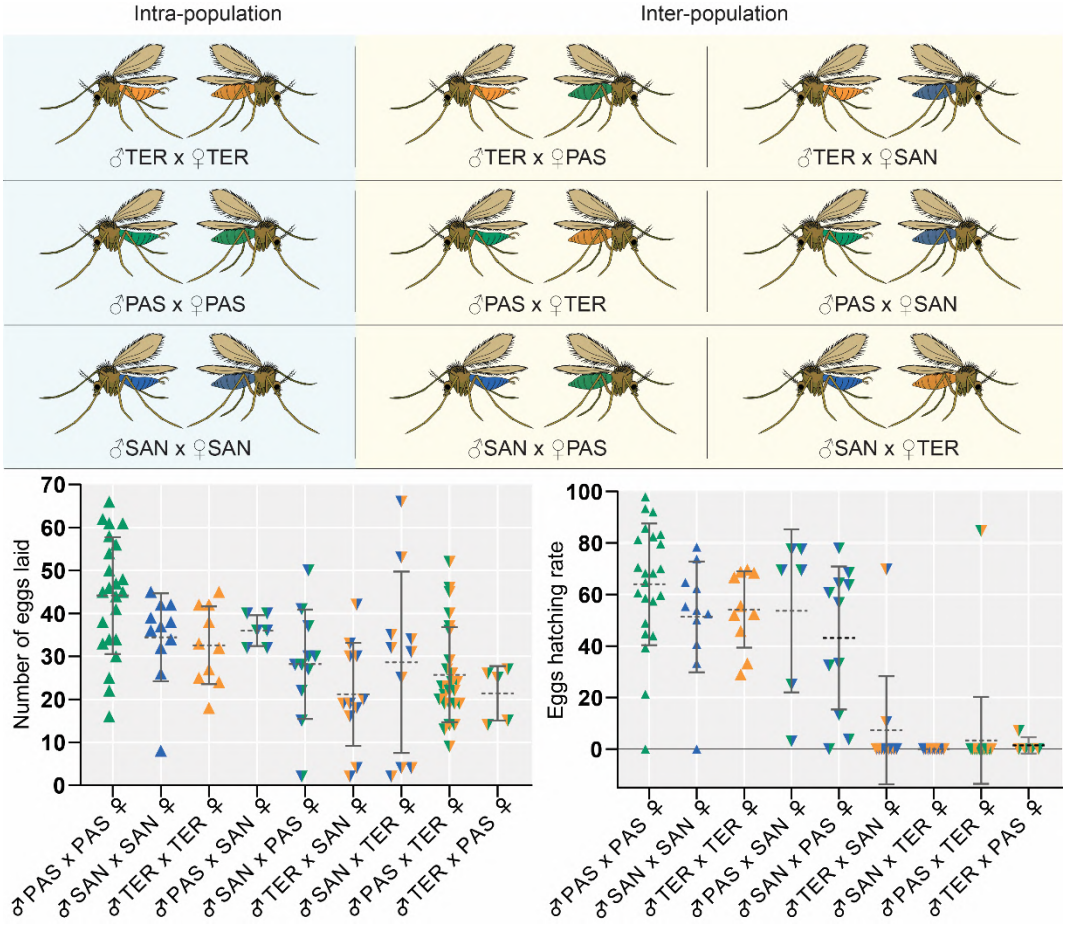
♀TER cross (Table 1). Similarly, in crosses between Passira and Teresina populations, low egg hatching rates per female were observed from for ♂PAS x ♀TER (3.4%; 95% CI: 0.0–10.4), and ♂TER x ♀PAS (1.4%; 95% CI: 0– 5.4). The egg hatching rate varied significantly ($F_{(5, 62)} = 12.81$, $P < 0.0001$) between inter-population crosses (Table 2 and Figure 1).

Table 2. One-way ANOVA tests for life cycle attributes of the crosses between allopatric populations of *Lutzomyia longipalpis* s.l.

Life cycle trait	Intra-population	Inter-population	All crosses
Number of eggs per female	$F_{(2, 41)} = 4.38$, $P = 0.019$	$F_{(5, 62)} = 1.30$, $P = 0.275$	$F_{(8, 103)} = 5.32$, $P < 0.0001$
Egg hatching rate	$F_{(2, 41)} = 1.57$, $P = 0.220$	$F_{(5, 62)} = 12.81$, $P < 0.0001$	$F_{(8, 103)} = 23.65$, $P < 0.0001$
Development time (BM to 1st adult)	$F_{(2, 41)} = 0.28$, $P = 0.7588$	$F_{(4, 16)} = 17.72$, $P < 0.0001$	$F_{(7, 57)} = 44.69$, $P = 0.08$

Note: BM = blood meal

Figure 1. Schematic representation of the crosses between populations of *Lutzomyia longipalpis* sensu lato. The number of eggs laid and the egg hatching rate per female are shown.



Note: PAS = Passira; SAN = Santarém; TER = Teresina; ♂= male; ♀ = female.

In order to analyse eventual differences in terms of life cycle, we followed-up and compared the development times for each population and crosses. Table 3 summarizes the life cycle data of the three parental populations (Passira, Santarém and Teresina) and the hybrid offspring resulting from their crosses. No differences were found between the development times of the three parental populations ($F_{(2, 41)} = 0.28$, $P = 0.7588$) and all crosses ($F_{(7, 57)} = 44.69$, $P = 0.08$), but there is difference between hybrid crosses ($F_{(4, 16)} = 17.72$, $P < 0.0001$) (Table 2).

Table 3. Developmental time of individuals from allopatric populations of *Lutzomyia longipalpis* sensu lato and their crossbreed offspring.

Developmental Time in Days	[1]Passira F20 Mean (Sd)	[2]Santarém >F80 Mean (Sd)	[3]Teresina F18 Mean (Sd)	[4]♂PAS x ♀SAN Mean (Sd)	[5]♂SAN x ♀PAS Mean (Sd)	[6]♂TER x ♀SAN Mean (Sd)	[7]♂PAS x ♀TER Total [a]	[8]♂TER x ♀PAS Total [a]
Blood meal to oviposition	7.2 (1.5)	7.6 (1.6)	7.3 (1.6)	9.3 (2.2)	9.6 (1.2)	11.0 (1.73)	7.0	7.0
Oviposition to egg hatching	5.6 (1.4)	5.3 (1.3)	5.9 (1.4)	6.7 (2.2)	6.8 (1.2)	5.3 (1.53)	4.0	4.0
1st instar	5.5 (0.6)	5.5 (0.5)	5.4 (0.5)	5.7 (0.5)	5.4 (0.5)	5.0 (0.0)	4.0	5.0
2nd instar	4.4 (0.8)	4.3 (0.8)	4.4 (1.0)	4.3 (0.5)	5.5 (1.2)	4.3 (0.58)	4.0	4.0
3rd instar	4.7 (0.9)	5.6 (1.6)	5.1 (1.4)	5.3 (0.5)	6.2 (1.8)	5.7 (1.15)	5.0	3.0
4th instar	7.7 (1.2)	6.9 (0.7)	7.4 (1.5)	8.0 (0.9)	8.7 (1.2)	9.3 (1.15)	5.0	6.0
Pupa	9.7 (0.5)	9.7 (0.5)	9.6 (0.5)	10.3 (0.6)	10.4 (1.5)	9.0 (1.0)	10.0	9.0
Oviposition to 1st adult	31.1 (2.1)	31.0 (2.1)	31.3 (2.2)	35.3 (3.1)	37.2 (1.5)	33.7 (4.5)	27.0	29.0
Oviposition to 1st male	31.1 (2.1)	31.0 (2.1)	31.3 (2.2)	35.3(3.1)	37.2 (1.5)	32.0 (2.8)	27.0	- [b]
Oviposition to 1st female	32.3 (2.8)	31.6 (2.2)	32.7 (2.9)	36.2 (2.2)	38.2 (0.8)	34.0 (4.6)	27.0	29.0
Mean generation time (BM to 1st adult)	39.5 (1.4)	39.5 (1.4)	39.1 (1.4)	44.7 (1.0)	47.7 (1.6)	44.7 (3.2)	34.0	36.0

Note: BM = blood meal; PAS = Passira; SAN = Santarém; TER = Teresina; ♂= male; ♀ = female.
Number of female's progeny: [1]*n* = 23; [2] *n* = 11; [3]*n* = 10; [4]*n* = 6; [5]*n* = 11; [6]*n* = 11; [7]*n* = 25; [8]*n* = 5.
[a] The offspring consisted of a single individual (female)
[b] a single female emerged

Discussion

The possibility of reproductive isolation between *L*. *longipalpis* s.l. populations have indicated the existence of different taxa under the name *L*. *longipalpis* (Ward et al. 1983, 1988; Lanzaro et al. 1993; Souza et al. 2008). Our crossbreeding experiments corroborate this hypothesis and indicate an asymmetric reproductive isolation between the populations from Santarém (1S) and Teresina (1S). In the same way, crosses between populations from Passira (2S) and Teresina (1S) resulted in significantly low egg hatching rates in both directions. On the other hand, we found no difference in terms of viability of larvae in crosses between Passira (2S) and Santarém (1S) populations. As a matter of fact, these results suggest the existence of a reproductive barrier between Teresina (1S) and Passira (2S) as well as between Teresina (1S) and Santarém (1S) populations. Similar results were found between sympatric populations (1S x 2S) of *L*. *longipalpis* s.l. from Sobral (Ward et al. 1983) and Venezuelan *L*. *longipalpis* s.l. population and *Lutzomyia pseudolongipalpis* (Arrivillaga et al. 2009). Crosses of males of *L*. *pseudolongipalpis* with females of *L*. *longipalpis* s.l. resulted in an inviable offspring, with no egg hatching. However, crosses between females of *L*. *pseudolongipalpis* and males of *L*. *longipalpis* s.l. resulted in a viable offspring. It is interesting to note that *L*. *pseudolongipalpis* is a *bona fide* species within the *L*. *longipalpis* complex (Arrivillaga and Feliciangeli 2001), but there is no a complete reproductive isolation with *L*. *longipalpis* s.l. Incidentally, at least other four nominal species compose the *L*. *longipalpis* complex (Sousa-Paula et al. 2021a), but crossbreeding experiments involving these other species have not been conducted as yet. In fact, few crossbreeding studies have been carried out with phlebotomine sand flies (dos Reis and Alevi 2020). A pivotal condition to perform such studies is rearing colonies in the laboratory, which is a complex, time-consuming and non-trivial task (Volf and Volfova 2011; Lawyer et al. 2017).

We have recently analysed the population genetic structure and phylogenetic relationship of these same three populations analysed herein (Sousa-Paula et al. 2021b). Our genetic data disclosed the presence of two prominent clades, composed by Teresina (1S) (clade I), and Passira (2S)/Santarém (1S) (clade II) populations. In addition, they also suggested a limited interclade gene flow. The crossbreeding data showed in the present study fits with the genetic data previously published (Sousa-Paula et al. 2021b). Altogether, these two studies underpin a limited capacity to

crossbreed these two groups of *L*. *longipalpis* s.l., which substantially adds weight to the hypothesis of distinct species in this species complex.

Furthermore, data suggest that the two clades observed in our genetic analyses may be sorted by the copulatory song produced by the males (Sousa-Paula et al. 2021b). The clade I grouped with populations that genuinely produce a Pulse-type song, whereas the clade II grouped with populations singing a Burst-type song (Vigoder et al. 2015). It has been recently reported that the copulatory song type is a determinant factor for female insemination success (Vigoder et al. 2020). It has been elegantly demonstrated that mating of wingless males (no copulatory song production) with females from the same population has decreased significantly insemination rate. Remarkably, the insemination rate significantly increases if a copulatory-playback sound is played, even in absence of wings (Vigoder et al. 2020). In this perspective, the low egg hatching rate and the reproductive isolation between ♂SAN x ♀TER presented in the present study may be driven by the copulatory song that these populations may sing.

Souza et al. (2009) performed the only study comparing the life cycle of populations of *L*. *longipalpis* s.l. They showed data from recent colonized allopatric and sympatric populations (F1 generation). Significantly differences were reported between the populations of two groups that produce copulatory songs and sex-aggregation pheromones (Souza et al. 2009). In contrast, we found no significant difference between our parental populations and crossbreeding. It is worth mentioning that we used populations > F18, it is likely that these populations are adapted and selected to the conditions set out in the laboratory. Furthermore, the life cycle of phlebotomine sand flies may be influenced by several environmental factors, such as humidity, temperature, and, importantly, the immature and adult diet (Lawyer et al. 2017; Martins et al. 2021). Therefore, slight differences in terms of the life cycle could be normalized along with successive generations in the laboratory.

In conclusion, our data suggest the existence of a reproductive barrier between the populations of *L*. *longipalpis* s.l. analysed herein. In particular, populations from Passira and Santarém had no difference in terms of offspring viability. However, we reported an asymmetric reproductive isolation between the populations from Teresina (1S) and Santarém (1S), and extremely low egg hatching rate between Teresina (1S) and Passira (2S). Along with our previously published genetic data, our crossbreeding

experiments corroborate the hypothesis on the existence of different taxa with the populations of *L*. *longipalpis* s.l. studied herein.

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5 CONCLUSÕES

As três populações de *L. longipalpis* s.l. analisadas no presente trabalho compreendem dois grupos genéticos distintos que, eventualmente, podem representar diferentes espécies. Os dados de estrutura genética e inferência filogenética demonstram que a população de *L*. *longipalpis* s.l. de Teresina é um grupo diferente em comparação àquelas de Passira e Santarém. Portanto, essas populações são candidatas a serem reavaliadas no futuro e, eventualmente, podem ser descritas como espécies distintas.

Os experimentos de cruzamentos entre as populações confirmaram a existência de barreiras reprodutivas entre a população de Teresina comparada àquelas de Passira e Santarém. Há um isolamento reprodutivo assimétrico entre Teresina e Santarém, com híbridos somente em uma das direções. Em conjunto com os dados genéticos, o isolamento reprodutivo reforça a existência de espécies distintas.

Antecedendo qualquer ato de nomeação taxonômica para as espécies crípticas de *L*. *longipalpis* s.l., a designação de um neótipo é condição *sine qua non* para a definição de qual dessas entidades é a espécie originalmente descrita, isto é, *L*. *longipalpis* s.s. Futuramente, abordagens integrativas, incluindo dados genômicos e outras espécies do complexo *L*. *longipalpis* s.l., devem auxiliar na elucidação desse *conundrum* taxonômico, bem como na história evolutiva desse importante grupo de insetos. A definição das espécies crípticas dentro do complexo *L. longipalpis* s.l. é uma questão que pode estar além de interesses taxonômicos. Uma vez que estas recebam nomes válidos e ferramentas confiáveis para o seu diagnóstico estejam disponíveis, diferenças determinantes — tais como preferência alimentar e taxa natural de infecção, susceptibilidade a patógenos etc. — poderão ser avaliadas.

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APÊNDICE A — ARTIGOS PUBLICADOS

PLOS | NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Failure of the dog culling strategy in controlling human visceral leishmaniasis in Brazil: A screening coverage issue?

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Competing interests: FDT has received funding from Bayer Animal Health to conduct a clinical trial

Abstract

In the present study, we assessed the annual screening coverage (i.e., the percentage of dogs that are screened for anti-*Leishmania* antibodies annually) in the municipality of Sobral, Ceará state, Brazil. Data on the number of dogs screened during 2008–2017 (except 2010) were obtained from the Centre for Zoonoses Control of Sobral. The annual screening coverage during 2012–2017 was calculated. Data on human visceral leishmaniasis (VL) cases during 2008–2017 were compiled from the National Disease Notification System. Correlation analyses were performed to assess the correlation between canine and human data. During 2008–2017, 73,964 dogs (range, 0 to 13,980 dogs/year) were serologically screened and 2,833 (3.8%) were positive. The annual screening coverage during 2012–2017 ranged from 11.1% to 45.7%. There were no significant correlations between the number of dogs culled and the number of human VL cases, canine positivity and human VL incidence, number of dogs culled and human VL incidence, or between canine positivity and number of human VL cases. An inconsistent and relatively low annual screening coverage was found in the study area, with no dog being screened in 2010 due to the lack of serological tests. Our results highlight that many dogs potentially infected with *Leishmania infantum* have been virtually overlooked by public health workers in the study area, perhaps with a negative, yet underestimated, impact on the control of canine and human VL. Hence, the failure of the dog culling strategy in controlling human VL in Brazil may be due to the low screening coverage and low percentage of culled dogs, rather than the absence of associations between canine and human infections.

Author summary

The euthanasia of *Leishmania*-seropositive dogs has been recommended for controlling human visceral leishmaniasis (VL) in some countries where this zoonosis is endemic. We assessed the annual screening coverage (i.e., the percentage of dogs living in a given area that are screened for anti-*Leishmania* antibodies annually) in the municipality of Sobral, Ceará state, one of the main foci of human VL in Brazil. From 2008 to 2017, nearly 74,000

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Seasonal dynamics and rickettsial infection in free-living *Amblyomma dubitatum* in the Atlantic forest biome in north-eastern Brazil

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ABSTRACT

The genus *Amblyomma* is the most representative tick genus in Brazil and some species act as vectors of pathogenic organisms to animals and humans. Information on the seasonal dynamics of *Amblyomma* spp. as well as on rickettsial organisms infecting these ticks in some regions in Brazil is still fragmentary. Herein, we investigated the seasonal dynamics and rickettsial infections in *Amblyomma dubitatum* ticks collected in the Atlantic forest biome in north-eastern Brazil. Using carbon dioxide traps, ticks were collected monthly for two consecutive years. In total, 15,789 ticks were collected: 69 females (0.4%), 116 males (0.7%), 1,067 nymphs (6.8%), and 14,537 larvae (92.1%). All nymphs, females and males were identified as *A. dubitatum*, whereas larvae were identified as *Amblyomma* spp. Larvae were more frequent in summer (77% of the larvae collected), whereas nymphs were collected with similar frequency in summer (32.8%), autumn (30.0%) and spring (28.4%). Adults were more frequent in spring (47.6%). A total of 648 ticks (485 nymphs, 60 females, and 103 males) were tested by PCR for the *gltA* gene of *Rickettsia* spp. and 87 (13.4%; 95% CI: 10.9–16.3%) were positive. A consensus sequence (size, 350 bp) of 66 *gltA* gene sequences indicate that the organism detected herein is similar to *Rickettsia tamurae, Rickettsia monacencis* and *Rickettsia* sp. strain Pampulha. One of these positive samples was also positive for the *ompA* gene of spotted fever group rickettsiae, but attempts to sequence the amplicon were not successful. We also tested this sample by a PCR targeting the rickettsial *htrA* gene, but no amplification product could be detected. This study indicates that *A. dubitatum* may be a common tick in areas where capybaras are present in north-eastern Brazil, occurring during the whole year. It also suggests the circulation of a spotted fever group rickettsia in this *A. dubitatum* population, whose identity has yet to be determined.

1. Introduction

The genus *Amblyomma* is the most speciose tick genus in the Neotropical region, with approximately 54 species occurring in this region (Guglielmone et al., 2014; Nava et al., 2014a, 2014b; Krawczak et al., 2015; Martins et al., 2019). Of these, 33 (~61%) are found in Brazil (Dantas-Torres et al., 2019a; Martins et al., 2019). Some *Amblyomma* spp. can act as vectors of pathogenic organisms to animals and humans. For instance, Brazilian spotted fever (BSF) is a life-threatening tick-borne illness caused by the bacterium *Rickettsia rickettsii*, which is primarily transmitted by the tick *Amblyomma sculptum* (Dantas-Torres et al., 2012b; Gerardi et al., 2019; Luz et al., 2019). As most ixodid ticks in the Neotropical region (Nava and Guglielmone, 2013), *A. sculptum* is a generalist tick. It displays a catholic feeding behavior, with horses and capybaras (*Hydrochoerus hydrochaeris*) standing as some of its primary hosts in most of the zones where this tick occurs (Martins et al., 2016; Luz et al., 2020).

Evidence indicate that opportunities for pathogen transmission via generalist ticks may be higher in degraded habitats (Esser et al., 2019). In this regard, recent investigations carried out in anthropic and natural habitats indicate that some areas where BSF is endemic are characterized by much higher tick burdens on capybaras and in the environment, with a great predominance of *A. sculptum* (Luz et al., 2019; Nunes et al., 2019). In contrast, BSF-nonendemic areas are characterized by low tick burdens on capybaras and in the environment, with the slight predominance of *Amblyomma dubitatum* (Luz et al., 2019). Overall, this suggests

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PROTOZOOLOGY - SHORT COMMUNICATION

Molecular epidemiology and prevalence of babesial infections in dogs in two hyperendemic foci in Brazil

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Abstract

Babesial parasites are some of the most ubiquitous blood pathogens and consequently have considerable worldwide veterinary impact. Dogs living in the tropics are highly exposed to babesial parasites, particularly to *Babesia vogeli*. Limited data on the seroprevalence and molecular prevalence of *Babesia* spp. in dogs are available in Latin America. We conducted a cross-sectional study combining serological and molecular tests to estimate the seroprevalence and molecular epidemiology of *Babesia* spp. infections in dogs in two hyperendemic foci in Brazil. A total of 630 privately owned dogs (417 from Goiana municipality, Pernambuco state, north-eastern Brazil, and 213 from São Joaquim de Bicas municipality, Minas Gerais state, south-eastern Brazil) were sampled and molecularly and serologically tested for *Babesia* spp. Overall, 519 dogs (82.4%) presented detectable IgG antibodies against *Babesia* spp., and seropositivity was significantly higher in dogs older than 1 year. Molecularly, 34 dogs (5.4%) were positive for a ~ 200 bp fragment of the *18S rRNA* gene of *Babesia* spp. and 88 (14.0%) for a longer fragment (~ 450 bp) of the same gene of *Babesia* spp. and other protozoa. The *18S rRNA* gene sequences generated herein corresponded to *B. vogeli* ($n = 52$) or *Hepatozoon canis* ($n = 20$). This study confirms a high level of exposure to *B. vogeli* in two areas of Brazil and highlights that most of the dogs living in these areas are infected during the course of their life, reflected by increased seroprevalence in older dogs. Increased awareness and prevention of tick-borne protozoa infections in dogs from Brazil and Latin America are urgently needed.

Keywords Age · *Babesia* · Dogs · Molecular epidemiology · Risk factor · Seroprevalence

Introduction

Canine babesiosis is a clinically relevant disease of dogs, which affects disproportionately dogs living in the tropics (Panti-May and Rodríguez-Vivas 2020). Scientific evidence gathered during the past decades unravelled that dogs can be infected by an expanding number of recognized babesial species (Baneth et al. 2019, 2020; Penzhorn, 2020). Nonetheless, *Babesia vogeli* remains as the most widespread species globally, which may be partly explained by the vast geographical distribution of its tick vectors (Dantas-Torres et al. 2018).

Canine babesiosis is widely distributed and highly endemic in tropical regions (Maggi and Krämer 2019; Panti-May and Rodríguez-Vivas 2020). For instance, a review of molecular studies of babesial infections in dogs in Latin America and the Caribbean revealed that prevalence varies widely according

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Tick infestation on birds in an urban Atlantic Forest fragment in north-eastern Brazil

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Abstract
Birds are important hosts for various tick species, playing a significant role in their biological life cycle and dispersion. In this study, we investigated tick infestations on birds trapped in an urban remnant of Atlantic Forest in Pernambuco state, Brazil. From February 2015 to March 2017, 541 birds belonging to 52 species were trapped with mist nets and examined for ectoparasites. Birds trapped in the late successional forest were significantly more infested than birds trapped in the early successional forest. In the same way, ectoparasite infestation varied significantly according to bird weight and collection plot. Overall, 198 birds (36.6%) belonging to 27 species were parasitized by ectoparasites (i.e., ticks, lice and/or mites). Ectoparasites were effectively collected from 111 birds, of which 99 belonging to 20 species were infested by ticks ($n=261$), namely, *Amblyomma longirostre* (13 nymphs), *Amblyomma nodosum* (21 nymphs), *Amblyomma varium* (one nymph), and *Amblyomma* spp. (five nymphs and 221 larvae). Most of the ticks (>90%) were collected from Passeriformes. This study provides the second record of *A. varium* in Pernambuco state and confirms that birds, especially Passeriformes, are important hosts for larvae and nymphs of *Amblyomma* spp. in the Atlantic Forest biome of Pernambuco.

Keywords Birds · Ixodida · Ixodidae · *Amblyomma* · Passerines · Rainforest

Introduction

Ticks belong to the order Ixodida, which includes parasitiform acarines that are obligate blood feeders, in one or more of their developmental stages (Dantas-Torres and Otranto 2020). They are significant ectoparasites, being capable to cause direct damage to their hosts and to transmit various pathogens, including bacteria, protozoa, viruses, and

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Ticks on reptiles and amphibians in Central Amazonia, with notes on rickettsial infections

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Abstract
Reptiles and amphibians are exceptional hosts for different ectoparasites, including mites and ticks. In this study, we investigated tick infestations on reptiles and amphibians trapped in Central Amazonia, and also assessed the presence of rickettsial infections in the collected ticks. From September 2016 to September 2019, 385 reptiles (350 lizards, 20 snakes, 12 tortoises, and three caimans) and 120 amphibians (119 anurans and one caecilian) were captured and examined for ectoparasites. Overall, 35 (10%) lizards, three (25%) tortoises and one (0.8%) toad were parasitized by ticks (124 larvae, 32 nymphs, and 22 adults). In lizards, tick infestation varied significantly according to landscape category and age group. Based on combined morphological and molecular analyses, these ticks were identified as *Amblyomma humerale* (14 larvae, 12 nymphs, 19 males, and one female), *Amblyomma nodosum* (three larvae, one nymph, and one female), and *Amblyomma rotundatum* (four larvae, three nymphs, and one female), and *Amblyomma* spp. (103 larvae and 16 nymphs). Our study presents the first records of *A. nodosum* in the Amazonas state and suggests that teiid lizards are important hosts for larvae and nymphs of *A. humerale* in Central Amazonia. Moreover, a nymph of *A. humerale* collected from a common tegu (*Tupinambis teguixin*) was found positive for *Rickettsia amblyommatis*, which agrees with previous reports, suggesting that the *A. humerale-R. amblyommatis* relationship may be more common than currently recognized.

Keywords Amazonia · *Amblyomma* · Amphibians · Reptiles · *Rickettsia*

Introduction

Amphibians and reptiles are among the oldest and most diverse groups of tetrapods on Earth (Jetz and Pyron 2018; Uetz et al. 2021). They have a long evolutionary history and presently occupy a wide array of environments in virtually all zoogeographical regions of the world (Procheş and Ramdhani 2012; Jetz and Pyron 2018; Simões et al. 2018; Uetz

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ANEXO A — CERTIFICADO DE APROVAÇÃO DA COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Ministério da Saúde

FIOCRUZ
Fundação Oswaldo Cruz
Instituto Aggeu Magalhães

COMISSÃO DE ÉTICA NO USO DE ANIMAIS

Certificado de Aprovação

CERTIFICAMOS QUE O PROJETO INTITULADO **"Avaliação da competência vetorial, fecundidade e sobrevivência dos fenótipos 1S e 2S do complexo** *Lutzomyia longipalpis***"** protocolado sob nº **147/2019** pelo pesquisador **Filipe Dantas Torres** está de acordo com a Lei 11.794/2008 e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS do Instituto de Pesquisas Aggeu Magalhães/Fundação Oswaldo Cruz (CEUA/IAM). Na presente versão, este projeto está licenciado e tem validade até Março de 2022 com a finalidade de pesquisa científica. É responsabilidade do coordenador do projeto notificar à CEUA de quaisquer alterações em relação ao projeto. O coordenador concorda que nenhuma dessas mudanças serão implementadas antes de serem aprovadas pela CEUA/IAM.

Quantitativo de Animais Aprovados				
Espécie/Linhagem	**Quant. (total)**	**Sexo (idade e peso variados)**		**Origem**
Camundongo / Balb/c	**30**	♂		Biotério
Hamster/ *Mesocricetus auratus / Golden*	**60**	♂		Biotério
Ave / *Gallus gallus*	**4**		♀	Fazenda
Cão/ *Canis lupus familiaris*	**2**		♀	Clínica veterinária ou residências
Equídeo / *Equus caballus*	**2**		♀	Fazenda
Gato / *Felis catus*	**4**		♀	Clínica veterinária ou residências
Suíno /*Sus scrofa domesticus*	**2**		♀	Fazenda
Coelho /*Oryctolagus cuniculus*	**1**		♀	Biotério
Total	**105**			

Recife (PE, Brasil), 01 de novembro de 2019

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