INSTITUTO NACIONAL DE PESQUISAS DA AMAZÔNIA – INPA

PROGRAMA DE PÓS-GRADUAÇÃO EM ENTOMOLOGIA

Diversidade, dinâmica comportamental e aspectos ecológicos e entomológicos de espécies de *Anopheles* Meigen, 1818 (Diptera: Culicidae) em uma área endêmica de malária da Amazônia oriental brasileira.

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Manaus, Amazonas

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Orientadora: Dra. Vera Margarete Scarpassa.

Tese apresentada ao Instituto Nacional de Pesquisas da Amazônia como parte dos requisitos para a obtenção do título de Doutor em Ciências Biológicas, área de concentração em Entomologia.

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Tese defendida em 27 de agosto de 2021.

Banca examinadora:

Drª. Mirian Silva Rafael
Instituto Nacional de Pesquisas da Amazônia – INPA

Dr. Felipe Arley Costa Pessoa
Fundação Oswaldo Cruz – FIOCRUZ

Dr. Allan Kardec Ribeiro Galardo
Instituto de Pesquisas Científicas e Tecnológicas do Estado do Amapá – IEPA

Dr. Ronildo Baiatone Alencar
Fundação de Vigilância em Saúde – FVS

Dr. Ricardo Adaime da Silva
Empresa Brasileira de Pesquisa Agropecuária – EMBRAPA

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Sinopse:

Estudou-se a diversidade de *Anopheles* em uma área de transmissão de malária, localizada no município de Santana, Amapá, Brasil. Análises faunísticas e ecológicas, distribuições espaciais e temporais de formas imaturas e adulta foram avaliadas. Adicionalmente, para os adultos foram analisados os padrões comportamentais [endofília/exofília, antropofília/zoofília, índice de atração por mosquito/homem/hora (IAHH) e atividade horária hematofágica], fatores entomológicos [taxa de paridade (TP) e infectividade por *Plasmodium* spp.] e avaliadas a taxa mínima de infecção (TMI) e taxa de inoculação entomológica (TIE) mensal e anual. Os criadouros foram caracterizados quanto aos parâmetros ambientais e limnológicos. Os imaturos também foram analisados quanto aos parâmetros entomológicos [índice de larvas por homem/hora (ILHH), índice de positividade de formas imaturas (IP), índice de criação geral (ICG), índice de criação absoluto (ICA) e índice de criação relativo (ICR)].

Palavras-chave: Faunística, ecologia, entomologia, distribuição, infectividade, criadouros.

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Resumo

Os anofelinos pertencem ao filo Arthropoda, subfilo Hexapoda, classe Insecta, ordem Diptera, subordem Culicomorpha, família Culicidae, subfamilía Anophelinae e gênero *Anopheles*, possuem grande importância em saúde pública por serem transmissores da malária humana. Este estudo teve como objetivo elucidar a diversidade de espécies de *Anopheles* e os aspectos entomológicos envolvidos na transmissão da malária no Distrito da Ilha de Santana, situado no município de Santana, Estado do Amapá, Brasil. Os adultos foram coletados no período de janeiro de 2017 a dezembro de 2018. As coletas foram divididas em três categorias com finalidades diferentes: avaliar os níveis de endofília e exofília, o índice de atração por mosquito/homem/hora, a atividade horária hemotofágica, e os parâmetros de antropofília e zoofília. O Distrito da Ilha de Santana foi dividido em três áreas: Área Urbana, Área de Transição e Área Rural. Os adultos foram analisados quanto à composição faunística, ecológica, padrões comportamentais e fatores entomológicos. As coletas de imaturos de anofelinos foram executadas mensalmente de julho de 2019 a junho de 2020 em todos os criadouros em potencial identificados. Os habitats das larvas foram caracterizados estruturalmente quanto aos parâmetros ambientais e limnológicos e as larvas foram analisadas quanto à composição faunística, ecológica e parâmetros entomológicos. Foram aplicados teste *t* de Student e o substituto não-paramétrico Mann-Whitney, de acordo com a distribuição dos dados, além de Kruskal-Wallis para as comparações de média. Para os índices faunísticos, ecológicos, ademais para o estudo das distribuições espacial e temporal (adultos e imaturos) e para avaliar as variações na composição e a resposta das espécies (imaturos) às variações dos fatores físico-químicos da água foram utilizadas análises multivariadas. Ainda foram elaborados mapas de distribuição de espécies (adultos e imaturos) e mapa de densidade de Kernel (imaturos). Para as análises dos criadouros quanto aos parâmetros ambientais foram aplicados Modelos Lineares Generalizados (GLM). As estatísticas descritivas foram realizadas no Programa PAST, PAleontological STatistics versão 1.34 e no Programa BioEstat versão 5.0. Um total de 3.163 exemplares foram coletados (1.330 adultos e 1.833 imaturos). Dos adultos, 276 foram obtidos durante as coletas com duração de quatro horas, 169 durante as coletas de 12 horas e 885 durante as coletas realizadas em ambientes com humanos (antropofília) e animais (zoofília). *Anopheles darlingi* foi a espécie mais abundante durante as coletas dos adultos de quatro e 12 horas (57,08%) e quando verificada a SIMPER a que mais contribuiu para a similaridade existente dentro de cada grupo de amostras: para as três áreas de estudo (adultos) e para o meio ambiente (imaturos). Também foi o anofelino mais frequente no intradomicílio (26%), o mais antropofílico (I_A = 0,39), apresentou as maiores atividade horária hematofágica e atração por mosquito/homem/hora nas primeiras horas com alta taxa de paridade, e foi a única espécie positiva para *Plasmodium vivax*. *Anopheles darlingi* também apresentou características generalistas quanto a distribuição na área. Para as formas imaturas desta espécie, os tanques de piscicultura apresentaram maior abundância (89,03%), principalmente na estação seca (74,32%), quando ocorreu a maior insolação anual e uma das menores pluviosidades, coincidindo com o aumento dos casos de malária. O mapa de kernel revelou três regiões críticas de alta vulnerabilidade para a transmissão da malária no distrito da Ilha de Santana, todas concentradas nas áreas de transição e rural. *Anopheles albitarsis* s.l. coexistiu com *A. darlingi* durante todo o período amostral, ambas as espécies foram dominantes na área urbana, representando 76,00% do total coletado para está área. Há evidências suficientes de que os tanques de piscicultura são os principais criadouros das formas imaturas e são os responsáveis pela manutenção do *A. darlingi* no Distrito da Ilha de Santana, pois, independente do período sazonal, continuam proporcionando condições favoráveis ao desenvolvimento dos vetores.

Palavras-chave: fauna, ecologia, padrões comportamentais, parâmetros entomológicos, parâmetros limnológicos, *Plasmodium* spp.

Abstract

Anophelines belong to the phylum Arthropoda, subphylum Hexapoda, class Insecta, order Diptera, suborder Culicomorpha, family Culicidae, subfamily Anophelinae, and genus *Anopheles.* They are of major importance for public health because they transmit human malaria. This study was aimed at characterizing the diversity of *Anopheles* species and examining the entomological aspects involved in malaria transmission in the district of Ilha de Santana, located in the municipality of Santana, state of Amapá, Brazil. Adults were collected from January 2017 to December 2018. The collections were divided into three categories with different goals: to evaluate endophilic and exophilic levels, the index of attraction as mosquito/human/hour, hourly biting activity, and aspects of anthropophily and zoophily. The district of Ilha de Santana was divided into three areas: urban, transition, and rural. Adults were analyzed regarding faunistic and ecological composition, behavioral patterns, and entomological factors. Collections of anopheline larvae were carried out monthly from July 2019 to June 2020 in all potential breeding sites identified. Larval habitats were structurally characterized based on environmental and limnological parameters, while larvae were analyzed based on faunistic and ecological composition and entomological parameters. The Student's t-test and the corresponding non-parametric Mann-Whitney test were performed according to the distribution of the data, in addition to the Kruskal-Wallis test for comparing means. Faunistic and ecological indices, spatial and temporal distributions (adults and immatures), and species composition and response (immatures) to changes in physical-chemical factors in the water were evaluated with multivariate analyses. Species distribution (adults and immatures) and Kernel density maps (immatures) were also created. Generalized Linear Models (GLM) were used to analyze environmental parameters of breeding sites. Descriptive statistics were obtained with the software PAST, PAleontological *STatistics* version 1.34 and BioEstat version 5.0. Overall 3,163 specimens were collected (1,330 adults and 1,833 immatures). Of adults, 276 were sampled in 4-h collections, 169 in 12-h collections, and 885 during collections performed in environments with the presence of humans (anthropophily) and animals (zoophily). *Anopheles darlingi* was the most abundant species in 4 and 12-h collections of adults (57.08%), and based on SIMPER, the one that most contributed for the similarity within each group of samples for the three study areas (adults) and for the environment (immatures). It was also the most frequent anopheline inside dwellings (intradomiciliary) (26%), the most anthropophilic ($I_A = 0.39$), with the highest hourly biting activity and index of attraction as mosquito/man/hour in the first hours with high parity rate, and the only positive species for *Plasmodium vivax*. *Anopheles darlingi* exhibited generalist characteristics regarding its distribution in the area. For immature forms of this species, abundance was higher in fish farming tanks (89.03%), especially in the dry season (74.32%) when annual insolation was highest and rainfall level was one of the lowest, coinciding with an increase in malaria cases. The kernel map revealed three critical regions of high vulnerability for malaria transmission in the district of Ilha de Santana, all concentrated in transition and rural areas. *Anopheles albitarsis* s.l. coexisted with *A. darlingi* throughout the sampling period, both species were dominant in the urban area, accounting for 76.00% of the total collected in this area. There is sufficient evidence that fish farming tanks are the main breeding grounds of immature forms and are responsible for maintaining *A. darlingi* in the district of Ilha de Santana, because regardless of the seasonal period, they continue to provide favorable conditions for the development of vectors.

keywords: fauna, ecology, behavioral patterns, entomological parameters, limnological parameters, *Plasmodium* spp.

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1. INTRODUÇÃO GERAL

Os anofelinos pertencem ao filo Arthropoda, subfilo Hexapoda, classe Insecta, ordem Diptera, subordem Culicomorpha, família Culicidae, subfamilía Anophelinae e gênero *Anopheles* Meigen, 1818 (Harbach, 2020; Wiegmann e Yeates, 2017). De acordo com a região, recebem popularmente a denominação de mosquito-prego, mosquito agulha, pernilongo, carapanã, bicuda, entre outros (Lorenz *et al*., 2018). Os mosquitos do gênero *Anopheles* possuem o desenvolvimento pós-embrionário por holometabolia, compreendendo os estágios de ovo, larva, pupa e adultos, sendo o estágio de larva subdividido em quatro estádios (WHO, 2015). Os imaturos são aquáticos, as larvas se alimentam de microrganismos planctônicos e matéria orgânica, enquanto os adultos são alados e de vida terrestre, alimentando-se de néctares vegetais (Williams e Pinto, 2012). Porém, as fêmeas são hematófagas, necessitam de proteínas encontradas no sangue dos vertebrados para a produção e desenvolvimento de ovos (Gouveia de Almeida, 2011).

Os imaturos de *Anopheles* se desenvolvem em coleções hídricas denominadas de criadouros, entre os habitats larvais estão incluídos: criadouros naturais com águas límpidas e com certa profundidade e vegetação aquática (flutuante), sombreadas e com baixo teor de sais e matéria orgânica; e também em habitats antropogênicos, como viveiros de peixes, lagos artificiais e barragens (Barros *et al*., 2011; Conde *et al*., 2015; Arcos *et al*., 2018). Dessa forma, são considerados potenciais para o desenvolvimento dos imaturos como: lagoas ou grandes lagos, remansos de rios, córregos, represas artificiais, alagados, manguezais, pântanos, valas de irrigação, entre outros (Williams e Pinto, 2012). Identificar os criadouros em potencial para o desenvolvimento de espécies de *Anopheles* é de suma importância epidemiológica no controle vetorial, portanto é essencial identificar e caracterizar esses criadouros, estruturalmente e relacioná-los aos parâmetros limnológicos (aspectos físicos e químicos da água dos criadouros) (Barros, 2012).

Os anofelinos possuem grande importância em saúde pública por serem principalmente transmissores do agente etiológico que causa a malária humana. A transmissão ocorre por meio da picada das fêmeas durante o repasto sanguíneo (Williams e Pinto, 2012). A malária é uma doença infecciosa, não contagiosa e de evolução crônica, com manifestações episódicas de caráter agudo. Devido sua característica endêmica, é responsável por muitos óbitos (WHO, 2016; Stevenson e Norris, 2017). A malária é provavelmente a doença parasitária mais antiga; há relatos em escritos chineses e egípcios de aproximadamente 3 mil anos a.C. da ocorrência de esplenomegalia e febre intermitente (Brasil, 2006; Tuteja, 2007). O agente infeccioso da malária foi descoberto somente em 1880, pelo cientista Charles Laveran com a identificação de

corpos claros nos eritrócitos, observações da formação de gametas machos e fêmeas e, posteriormente, o fenômeno da exoflagelação. A transmissão da malária por mosquitos foi somente comprovada em 1898, por Ronald Ross, ao estudar a malária aviária (Brasil, 2006; Tuteja, 2007).

A malária está confinada a áreas tropicais, principalmente em países pobres da África subsaariana, Ásia e América Latina, onde o controle da doença é agravado pela ausência de estruturas de saúde adequadas, as condições socioeconômicas precárias e a resistência às drogas normalmente usadas no combate ao parasito que provoca a doença, ademais às condições ambientais e climáticas dos países tropicais que favorecem a proliferação do mosquito *Anopheles* (Dutra, 2007; Lima e Guimarães, 2007). Atualmente, nas Américas, o Brasil juntamente com a Colômbia e Venezuela representa 86% dos casos estimados de malária (WHO, 2020). No Brasil, aproximadamente 99,8% dos casos estão concentrados na região amazônica (SIVEP/MS, 2020; WHO, 2020). Os estados que pertencem à Amazônia legal, onde estão incluídos os maiores números de casos da doença são: Acre, Amazonas, Amapá, Pará, Rondônia e Roraima (SVS, 2020).

Este estudo apresenta uma compilação detalhada envolvendo as formas adulta e imaturas de *Anopheles* em uma área endêmica de malária na Amazônia oriental, abordando a composição de espécies, aspectos ecológicos, distribuições espaço-temporal, padrões comportamentais, fatores entomológicos, caracterização estrutural e parâmetros limnológicos de habitats larvais. Ao todo foram gerados três manuscritos, distribuidos da seguinte forma: No primeiro, abordamos os padrões comportamentais das fêmeas de *Anopheles* envolvidas na transmissão da malária no local. No segundo, versamos sobre a composição, fauna, ecologia, distribuição espaço-temporal dos adultos e imaturos de espécies de *Anopheles*. E, por fim, discorremos sobre a caracterização estrutural, os parâmetros entomológicos e limnológicos de habitats das formas imaturas dos anofelinos. Os resultados deste estudo fornecerão subsídios para a implementação de medidas de controle mais eficazes e direcionadas, assim como o planejamento de técnicas de manejo para tanques de piscicultura, a fim de se alcançar as metas estabelecidas pela Organização Mundial de Saúde no combate e eliminação da malária.

1.1 Considerações gerais sobre a Malária

A malária é um dos maiores problemas de saúde pública no Brasil e no mundo, com ampla distribuição especialmente nas regiões tropicais e subtropicais e, no geral, está associada

a pessoas com condições econômicas baixa. É uma doença infecciosa, febril, aguda, sistêmica, não contagiosa, de transmissão vetorial e potencialmente grave, uma protozoose causada por parasitos pertencentes ao filo Apicomplexa, família Plasmodiidae e gênero *Plasmodium* (WHO, 2010; Neves, 2016). É conhecida popularmente como paludismo, febre palustre, impaludismo, maleita, febre intermitente, entre outros (Rey, 2001; CDC, 2013).

No momento, são conhecidas 156 espécies de *Plasmodium* causadoras de malária em diferentes hospedeiros vertebrados (CDC, 2013). Entretanto, apenas seis espécies são reconhecidas por parasitar humanos: *Plasmodium falciparum* (Welch 1897), *P. vivax* Grassi e Feletti 1890, *P. malariae* Feletti e Grassi 1889, *P. ovale curtisi* Sutherland *et al*. 2010, *P. ovale wallikeri* Sutherland *et al*. 2010 e *P. knowlesi* Sinton e Mulligan 1932 (Calderaro, 2013). Por divergências na sequência nucleotídica do gene da proteína Circunsporozoita (CS) em isolados de *P. vivax*, são descritas três variantes para essa espécie: VK 210, VK 247 e *P. vivax-like* (Rosenberg *et al.*, 1989; Qari *et al.*, 1993; Lim *et al.*, 2001). Entretanto, no Brasil é registrada a ocorrência de malária causada apenas pelas espécies de *Plasmodium*: *P. vivax*, *P. falciparum* e *P. malariae*, sendo as duas primeiras as principais causadoras da doença no país (Rey, 2001). Entretanto, *P. vivax* é a espécie mais prevalente, constituída por três variantes: *P. vivax* VK 210, *P. vivax* VK 247 e *P. vivax-like*, sendo a variante *P. vivax* VK 210 responsável pelo maior número de infecções (Ohnishi, 2014). *Plasmodium ovale curtisi* e *P. ovale wallikeri* ocorrem apenas em regiões restritas do continente africano e *P. knowlesi* no sudeste da Ásia (Cox-Singh e Singh, 2008; Oguike *et al*., 2011).

Nas Américas, a prevalência das infecções ocorre pelo *P. vivax*, responsável por 76% dos casos (WHO, 2020). Embora menos frequente no continente americano, porém com elevados índices de óbitos, as formas graves e complicadas são causadas por *P. falciparum* (forma maligna) (WHO, 2020). A fase sintomática envolve mal-estar, cefaleia, cansaço, mialgia, calafrio, sudorese e fraqueza, as alterações no quadro clínico e no desenvolvimento deste protozoário no organismo do hospedeiro e a gravidade ocorrem de acordo com a espécie e a cepa infectantes, além do grau de imunidade do hospedeiro infectado (Andrade, 2005; SES/PB, 2019).

Em 2019, foram registrados cerca de 229 milhões de casos de malária em todo o mundo, um número ligeiramente maior comparado com 2018 (228 milhões) (WHO, 2020). Atualmente, a taxa de incidência da malária global está em níveis entre 57 casos /1.000 população em risco, sendo o maior número de casos, concentrado na África com 94% da malária global (WHO, 2020). Nas Américas, ocorreu uma redução em 40% no número de casos de malária (de 1,5

milhão para 0,9 milhão), mas ainda assim estima-se que 139 milhões de pessoas estão sob o risco de contrair a doença nesse continente (WHO, 2020).

Em 2019, somente o Brasil foi responsável por 20% dos casos de malária registrados nas Américas. Contuto, em 2020, apresentou uma redução no número de casos de malária com 135.658 casos; desses, 132.078 ocorreram na região amazônica (SIVEP/MS, 2020; WHO, 2020). Em 2019, o estado do Amapá registrou 15.246 casos com o maior número nos municípios de Mazagão, Porto Grande e Santana (SIVEP/MS, 2020). No município de Santana, em 2019, foram registrados 1.872 casos com um Índice Parasitário Anual (IPA) de 15,7 casos /1.000 habitantes. No mesmo ano, no Distrito da Ilha de Santana o IPA foi de 103,4 casos /1.000 habitantes sendo considerada uma área de alto risco de transmissão de malária (IPA $\geq$ 50) (SIVEP/MS, 2020).

Estudos de variáveis epidemiológicas implicadas na transmissão de malária, que elucidem a taxonomia associada aos padrões comportamentais, às variações temporais e de infectividade de espécies de *Anopheles* são incipientes no Estado do Amapá. Embora vários estudos tenham sido realizados em áreas específicas no referido Estado, poucos estudos avaliaram o perfil epidemiológico em uma área com transmissão da malária. Considerando a elevada riqueza de espécies de anofelinos e a complexidade das espécies envolvidas na transmissão da malária, estudos adicionais são extremamente necessários.

No município de Santana, dentre os poucos estudos realizados, este é pioneiro sobre a dinâmica populacional dos anofelinos na Ilha de Santana. Um estudo completo envolvendo aspectos ecológicos de distribuição, padrões comportamentais, longevidade, infectividade e identificação molecular de espécies de importância epidemiológica, fornece o conhecimento da dinâmica populacional, de acordo com a flutuação temporal, dispondo de informações que auxiliarão em estratégias mais eficazes no controle populacional dos vetores da malária.

Nos últimos anos, a Ilha de Santana tem sido assolada por vários surtos de malária, e tem sido considerada uma área de transmissão em potencial em função das características ambientais do local: ambiente relativamente preservado e com ampla cobertura vegetal. Portanto, são essenciais estudos que contribuam para o entendimento dos aspectos ecológicos e entomológicos de anofelinos, como forma de entender os mecanismos de transmissão da malária e a relação entre as populações de mosquitos, o ambiente e os hábitos das populações humanas local.

Para fins epidemiológicos e implantação e efetivação de medidas de controle vetorial, é relevante o conhecimento das espécies de *Anopheles* de uma região onde a malária é endêmica. A obtenção de dados, que estabeleçam os padrões comportamentais das espécies constituem

parâmetros fundamentais que poderão nortear as medidas a serem adotadas para evitar o contato homem-vetor. As variações de comportamento são indicadoras da existência de variedades geográficas populacionais ou mesmo de espécies crípticas.

1.2 Fauna, Ecologia e Distribuição espaço-temporal dos principais vetores da malária na Amazônia brasileira

Os anofelinos possuem distribuição mundial, ocorrendo em áreas temperadas, subtropicais e tropicais, exceto na maioria das ilhas do Oceano Pacífico (Harbach, 2020). Atualmente, em todo o mundo são registradas aproximadamente 480 espécies de *Anopheles* e cerca de 70 podem transmitir o impaludismo (WHO, 2015; Harbach, 2020). Este gênero está classificado em oito subgêneros: *Anopheles* Meigen 1818, *Cellia* Theobald 1902, *Christya* Theobald 1903, *Stethomya* Theobald 1902, *Nyssorhynchus* Blanchard 1902, *Kerteszia* Theobald 1905, *Lophopodomyia* Antunes 1937 e *Baimaia* Harbach, Rattanarithikul e Harrison 2005 (Harbach, 2017).

Na África, continente mais afetado pela malária, as principais espécies incriminadas como vetores pertencem ao complexo *Anopheles gambiae* s.s. Giles, 1902 (Akogbéto *et al*., 2018). Nas Américas a zona malarígena está dividida em três regiões: a primeira ao norte do Planalto Mexicano; a segunda envolvendo toda a América Central e Antilhas até a costa norte da Colômbia e Venezuela; e a terceira compreendendo parte do Continente sul-americano (Rey, 2001).

A transmissão da malária na América do Sul ocorre em todos os países amazônicos (Brasil, Bolívia, Peru, Equador, Colômbia, Venezuela, Guiana, Suriname e Guiana Francesa) (Brasil, 2020a). No Brasil já foram reportadas cerca de 69 espécies de *Anopheles*; destas, mais de 33 ocorrem na Amazônia brasileira e mais de 23 espécies já foram relatadas no estado do Amapá (Deane *et al*., 1948; Deane *et al*, 1971; Tadei *et al*., 1998; Bergo *et al*., 2007; Barbosa e Souto, 2011; Ferreira *et al*., 2013; Galardo *et al.*, 2015; Barbosa *et al.*, 2016; Walter Reed Biosystematics Unit, 2020). As espécies com ocorrência no país estão distribuídas em cinco subgêneros, com ausência de representantes apenas para *Cellia*, *Christya* e *Baimaia* (Harbach, 2017). No entanto, as espécies que apresentam maior importância epidemiológica pertencem aos subgêneros *Kerteszia* e *Nyssorhynchus*, sendo o último responsável pelas principais espécies envolvidas na transmissão da malária humana na Amazônia, incluindo *Anopheles darlingi* Root, 1926 e alguns membros dos complexos Albitarsis e Nuneztovari (Hiwat e Bretas, 2011).

Anopheles darlingi é considerado o principal vetor da malária humana na Amazônia, possui alta capacidade vetorial, pois é altamente susceptível aos plasmódios humanos (Santos *et al*., 2009; Sallum *et al*., 2019). É um mosquito predominantemente sul-americano; no Brasil, só não é encontrado nas áreas secas do Nordeste, no extremo Sul (abaixo da foz do rio Iguaçu) e nas áreas de elevada altitude (Consoli e Lourenço-de-Oliveira, 1994; Forattini, 2002), possuindo vasta distribuição na região amazônica (Sinka *et al*., 2012). Para essa espécie, no Brasil, já foram identificadas três subpopulações geneticamente estruturadas, (1) composta por indivíduos da Mata Atlântica (província) (sudeste); (2) Floresta do Paraná (província) (oeste da Mata Atlântica); (3) região amazônica (Emerson *et al.*, 2015); no entanto, até o momento é reconhecido como única espécie. É o anofelino mais antropofílico e de comportamento endófilico mais acentuado, porém essa espécie pode ser preferencialmente exofílica, apresentando ecletismo e oportunismo na preferência e no comportamento alimentar, mesmo em baixa densidade (Santos *et al*., 2009; Barbosa, 2012). Quanto à distribuição temporal, alguns estudos revelam o aumento da abundância de *A. darlingi* no início e no final da estação chuvosa, ou seja, no período de transição (Klein e Lima, 1990; Tadei *et al.*, 1998). Entretanto, no estado do Amapá, essa espécie tem apresentado maior frequência na estação seca, desempenhando uma distribuição estacional unimodal (Barbosa e Souto, 2011; Barbosa *et al.*, 2014).

Membros do complexo Albitarsis estão distribuídos em todo o continente sul-americano, com ampla distribuição que abrange desde o leste da Cordilheira dos Andes até o norte-nordeste da Argentina, Uruguai e Brasil (Sinka *et al*., 2012). Alguns membros desse complexo desempenham papel de vetor secundário e em alguns momentos assumem o papel de vetor principal em algumas regiões da Amazônia brasileira, como descreveram Póvoa *et al.* (2001), Conn *et al*. (2002) e Barbosa *et al*. (2014), em estudos realizados no estado do Amapá. A partir do uso de métodos moleculares, foram identificadas para este complexo dez categorias específicas: A – *A. albitarsis* s.s. Lynch-Arribálzaga, 1878; B – *A. oryzalimnetes* Wilkerson e Motoki, 2009; C – *A. marajoara* Galvão e Damasceno, 1942; D – *A. deaneorum* Rosa-Freitas, 1989; E – *A. janconnae* Wilkerson e Sallum, 2009; *A. albitarsis* F; *A. albitarsis* G; *A. albitarsis* H; *A. albitarsis* I e provavelmente uma nova linhagem, *A. albitarsis* J – ainda não descritas (Motoki *et al*., 2021). *Anopheles marajoara* e recentemente, *A. janconnae*, reportadas para o estado do Amapá, apresentam distribuição temporal com os maiores picos nas estações chuvosa e seca, exibindo um padrão estacional bimodal (Barbosa *et al.*, 2014; Motoki *et al*., 2021).

Anopheles nuneztovari s.l. também compreende a um complexo de espécies, constituído por: *A. nuneztovari* s.s. Gabaldón, 1940; *A. goeldii* Rozeboom e Gabaldon, 1941; *A. dunhami* Causey, 1945 e *A. nuneztovari* citótipo A (Sant'Ana *et al*., 2015; Santos e Santos, 2019). Os

membros desse complexo possuem ampla distribuição, sendo encontrados desde o leste do Panamá até ao norte da América do Sul e em toda a bacia amazônica, ocorrendo na Bolívia, Brasil, Colômbia, Equador, Guiana, Peru e Venezuela (Sinka *et al.*, 2010). Conforme Scarpassa *et al*. (2016), na Amazônia brasileira esta espécie pode consistir de pelo menos três taxons distintos de origem evolutiva muito recente. *Anopheles goeldii* pode estar envolvida na transmissão da malária no estado do Amapá (Galardo *et al*., 2007).

Outros anofelinos com ampla distribuição na Amazônia brasileira, considerados vetores locais, auxiliares, acidentais ou potenciais por já terem sido encontrados naturalmente infectados, embora alguns autores não atribuam importância epidemiológica, são: *Anopheles braziliensis* Chagas, 1907, *A. oswaldoi* s.l. (Peryassú, 1922), *Anopheles triannulatus* s.l. (Neiva e Pinto, 1922), *Anopheles evansae* (Brethes, 1926), *Anopheles galvaoi* Causey Deane e Deane, 1945 e *Anopheles benarrochi* Gabaldón, Cova-Garcia e Lopez, 1941 (Deane, 1986; Tadei *et al.*, 1993; Consoli e Lourenço-de-Oliveira, 1994; Flores-Mendoza *et al*., 2004). Essas espécies possuem hábitos preferencialmente zoofílicos e são, geralmente, pouco suscetíveis ao *Plasmodium vivax* Grassi e Feletti, 1890 e *P. falciparum* Welch, 1897 (Deane *et al.*, 1948).

Na região Neotropical, é comum observar uma riqueza de espécies com padrões de distribuição diversos (Tadei *et al.*, 1993). Essa variedade nos padrões de distribuição pode ser explicada pelos fatores influenciadores do clima local, impactos criados por corpos de água, topografia, heterogeneidade ambiental, qualidade e quantidade de recursos disponíveis e a interação entre organismos (D'Avanzo, 2004; Rebêlo *et al.*, 2007; Raven *et al*., 2014; Hackbart *et al*., 2015; Bear *et al*. 2016; De-Carli *et al*. 2017). A compreensão dos parâmetros, presença do vetor, densidade, contato homem-vetor e o número de casos de malária autóctones associado ao ecossistema investigado contribuem para melhor entender a dinâmica de transmissão da malária em diferentes ecossistemas (Zimmerman, 1992; Camargo *et al.*, 1994; Rubio-Palis e Zimmerman, 1997; Lounibos e Conn, 2000; Rosa-Freitas *et al.*, 2007).

Barbosa *et al.* (2014), investigando os anofelinos no Amapá, evidenciaram picos de densidade na estação seca, semelhante ao observado por Póvoa *et al.* (2009) no município de Juruti (Pará), em área com pluviometria média similar, igualmente por Tadei *et al.* (1988; 1993; 1998) na zona rural de Ariquemes (Rondônia) e por Silva *et al*. (2010), quando *A. darlingi* foi mais frequente na estação seca e, em seguida, teve sua frequência reduzida. As mudanças nas condições climáticas podem ocasionar flutuações de temperatura ou modificações no padrão da pluviosidade que podem indiretamente interferir na capacidade vetorial, além de alterarem a disponibilidade de criadouros (Bomblies e Eltahir, 2009; Murdock *et al*., 2016).

1.3 Dinâmica comportamental hematofágica

O comportamento das espécies envolvidas na transmissão da malária influência no padrão epidemiológico local (Barbosa *et al*., 2016). A mesma população pode apresentar variações comportamentais em função de alterações externas, aumentando a complexidade da dinâmica de transmissão da doença e elevando o potencial de transmissão dos vetores (Voorham, 2002; Santos *et al.*, 2009). Estas alterações externas podem ser ecológicas, ambientais e demográficas. As ações antrópicas, como desmatamento, criação de novos habitats para os imaturos, podem ocasionar uma pressão seletiva que induz as populações de vetores a se adaptarem às novas circunstâncias (Kuwabara, 2008). Outra mudança é quanto ao comportamento de endofília para exofília, resultado da borrifação residual intradomiciliar (BRI), selecionando no vetor resistência comportamental e/ou fisiológica aos inseticidas utilizados (Glunt *et al*., 2015; Ranson e Lissenden, 2016).

Os padrões comportamentais dos anofelinos são determinados pela composição de espécies de cada área, a densidade, as interações interespecíficas, o comportamento hematofágico, a intensidade de contato com o homem (considerando o comportamento social e cultural das populações humanas), a susceptibilidade à infecção por *Plasmodium* spp. e a longevidade das fêmeas (Tadei, 1993; Tadei *et al.*, 1998; Begon *et al.*, 2007). O hábito e o padrão alimentar das fêmeas de anofelinos fornecem informações essenciais para o entendimento do comportamento de espécies em áreas de transmissão da malária e auxiliam no desenvolvimento e planejamento de estratégias eficazes de controle (Brasil, 2019).

A maioria dos anofelinos é caracterizada principalmente por serem exofílicos, zoofílicos e crepusculares, que na ausência dos seus hospedeiros preferenciais ou nas épocas de elevada densidade, podem realizar a hematofagia no homem e infectar-se com gametócitos de plasmódios em áreas cuja a endemicidade é desencadeada e mantida por *A. darlingi* (Consoli e Lourenço-de-Oliveira, 1994).

1.3.1. Níveis de endofília e exofília

A estratégia utilizada pelos anofelinos para encontrar suas fontes sanguíneas inicia com o rastreamento ativo combinada com a espera em locais frequentados pelos hospedeiros preferenciais. Esses lugares podem ser representados pelos domicílios humanos e abrigos de animais domésticos, podendo a atividade hematófaga ser endofílica e/ou exofílica (Forattini, 2002). As fêmeas são atraídas pela fonte alimentar através de estímulos variados, incluindo: correntes de convecção que se formam a partir do hospedeiro, presença de dióxido de carbono

(CO_2), ácido lático, temperatura e umidade corporal (Zwiebel e Takken, 2004; Lorenz *et al*., 2018). A avaliação dos níveis de endofília e exofília auxilia na avaliação da capacidade vetorial de uma espécie e constitui característica relevante em estudos de populações de *Anopheles*, pois revelam os padrões da dinâmica de transmissão da malária (Tadei *et al.*, 1993; 1998).

Anopheles darlingi apresenta alta plasticidade e ecletismo quanto ao comportamento hematofágico, possivelmente, resultante da ampla distribuição geográfica (Prussing *et al*., 2018; Prado *et al*., 2019) e também consequência da elevada variabilidade genética das populações desta espécie (Chu *et al*., 2020). Além disso, é o anofelino mais endofílico das Américas, embora em diferentes localidades da Amazônia tem sido reportado comportamento predominantemente exofílico (Galardo, 2010; Barbosa *et al*., 2016).

Os anofelinos membros do complexo Albitarsis apresentam comportamento preferencialmente exófilo, porém por se tratar de um complexo de espécies, esse comportamento pode variar de acordo com a região geográfica (Galardo, 2010). Outro complexo de espécies com importância epidemiológica para o estado do Amapá, é *A. nuneztovari* s.l. que apresenta comportamento distinto (Calado *et al.*, 2008; Fajardo Ramos *et al.*, 2008; Mirabello e Conn, 2008; Scarpassa e Conn, 2011). Alguns membros desse complexo exibem comportamento exofílico e zoofílico no Brasil, Suriname e Equador; enquanto outra espécie *A. nuneztovari* s.s. apresenta característica predominantemente endofílica e antropofílica e ocorre particularmente em áreas da Venezuela e Colômbia (Conn, 1990; Rubio-Palis e Curtis, 1992; Calado *et al.*, 2008; Fajardo Ramos *et al.*, 2008; Mirabello e Conn, 2008; Scarpassa e Conn, 2011) e é considerado o principal vetor da malária naqueles países.

Apesar de alguns anofelinos apresentarem um padrão comportamental eminente, vários fatores podem influenciar nos hábitos em determinada localidade ou região. Características próprias da localidade podem ainda influenciar na diversidade e abundância de algumas espécies de anofelinos e, consequentemente, na dinâmica de transmissão da malária (Tadei *et al.*, 1993).

1.3.2. Atividade hematofágica

Outro aspecto essencial com relevância epidemiológica é estabelecer a atividade hematofágica e os horários preferenciais dos anofelinos vetores (Maciel e Missawa, 2012). Os períodos de atividade hematofágica dos anofelinos são crepusculares (amanhecer e entardecer) e/ou noturnos, com picos nas primeiras horas da noite (Neves, 2007; SES/PB, 2019). Entretanto, observa-se uma atividade crepuscular acentuada nas primeiras horas da noite para a maioria das

espécies, embora hajam espécies que exercem a hematofagia durante todo o período noturno (Neves, 2007; SES/PB, 2019).

Cada espécie possui um padrão de atividade hematofágica (crepuscular ou noturno) que pode ser alterado conforme o período sazonal, pluviosidade, densidade populacional do anofelino e as condições da área (naturais ou alteradas) (Tadei *et al.*, 1988; Lourenço-de-Oliveira *et al.*, 1989). Nas populações de *A. darlingi* ocorre plasticidade intrapopulacional quanto à atividade hematofágica conforme a região (Santos *et al.*, 2005). Em estudos realizados na Amazônia brasileira (Acre, Amapá, Pará e Roraima), *A. darlingi* tem exibido atividade hematofágica contínua, com padrões unimodais e bimodais ou até multimodais (Silva-Vasconcelos *et al.*, 2002; Voorham, 2002; Barros *et al.*, 2007; Moutinho *et al.*, 2011). Voorham (2002) e Barbosa *et al*. (2016), no Amapá, encontraram uma variabilidade na atividade hematofágica ao longo da noite, com vários picos (padrão multimodal). Essa espécie não apresenta padrões bem definidos (Gil *et al*., 2015; Lainhart *et al*., 2015; Moreno *et al*., 2015; Tadei *et al*. 2017; Saavedra *et al*., 2019), podendo ocorrer uma variação intrapopulacional tão ampla quanto a variação interpopulacional (Voorham, 2002). Por outro lado, *A. albitarsis* s.l., *A. nuneztovari* s.l., *A. triannulatus* s.l. e *A. oswaldoi* s.l. apresentam picos mais regulares (Tadei *et al.*, 1983). *Anopheles albitarsis* s.l., comumente apresenta um padrão mais estável (Barbosa *et al*, 2016), com comportamento crepuscular vespertino (Voorham, 2002; Póvoa *et al.*, 2006). Igualmente, são relatados para *A. nuneztovari* s.l. atividade hematofágica com picos ocorrendo nos primeiros horários da noite (Barros *et al*., 2020).

1.3.3. Parâmetros de antropofília e zoofília

Existem populações de anofelinos que realizam a hematofagia em um grande número de animais, enquanto outras têm essa capacidade restrita a poucos ou mesmo a uma única espécie de hospedeiro, aqueles que se alimentam de sangue humano são denominados antropofílicos, enquanto aqueles que apresentam preferência pelo sangue de animais são zoofílicos (Forattini, 2002). O grau de antropofília é uma condição essencial para que uma espécie de anofelino seja considerada vetor de malária humana (Gouveia de Almeida, 2011), como se tem observado para *A. darlingi*. Além disso, em uma mesma espécie o grau de preferências hematófagicas pode variar conforme a região (Forattini, 2002).

Anopheles darlingi é considerado o anofelino mais antropofágico (Kiszewski *et al*., 2004) das Américas. O desmatamento e o processo de antropização exercem influência na

densidade local dessa espécie, que apresenta alta plasticidade adaptativa e um elevado grau de sinantropismo (Vittor *et al*., 2006; Gomes *et al*. 2008).

Por se tratarem de complexos de espécies com características comportamentais próprias é difícil afirmar a ocorrência de antropofília em grau acentuado e de maneira uniforme para todas as populações de *A. albitarsis* s.l. e *A. nuneztovari* s.l., ambas com ampla distribuição (Forattini, 2002; Sinka *et al.*, 2010). Em estudo realizado no oeste da Amazônia brasileira *A. albitarsis* s.l. foi descrito como predominantemente zoofílico (Oliveira-Ferreira *et al*., 1992). No Pará, na região de Tucuruí, *A. nuneztovari* s.l. foi encontrado em alta densidade e com acentuada antropofília, podendo desempenhar papel de vetor auxiliar na transmissão da malária naquela região (Rebêlo *et al.*, 1997; Tadei *et al.*, 1998), mas esta população pode representar uma nova espécie no complexo Nuneztovari, conforme constatado por Scarpassa *et al.* (2016), através do emprego de marcadores microssatélites e sequencias do gene *COI*.

Apesar de várias espécies de anofelinos exibirem tendência de zoofília (*A. braziliensis*, *A. nuneztovari* s.l., *A. oswaldoi* s.l., *A. triannulatus* s.l., entre outros), podem eventualmente realizar a hematofagia em humanos e se infectarem, tornando-se vetores acidentais (Consoli e Lourenço-de-Oliveira, 1994). O comportamento zoofílico de *A. triannulatus* s.l e *Anopheles intermedius* (Peryassú, 1908) sugere que essas espécies não possuem importância vetorial no estado do Amapá, embora em alta densidade possam contribuir para a transmissão do parasito (Zimmerman *et al.*, 2006; Barbosa, 2012).

1.4 Paridade

A partir da avaliação da taxa de paridade é possível verificar a capacidade vetorial de anofelinos e relacioná-la com a densidade, a proporção de picadas, o grau de antropofília, entre outros parâmetros (Deus e Kakitani, 2006). É possível avaliar a paridade das fêmeas de anofelinos pela observação das características dos filamentos das traqueólas ovariolares e dos ovaríolos, permitindo inferir sobre a sobrevivência da fêmea e a idade fisiológica a partir do número de oviposições (Kuwabara, 2008). As maiores taxas de paridade implicam em maior risco de transmissão da malária, as fêmeas oníparas apresentam maior longevidade, consequentemente, maior probabilidade de terem mantido contato com o parasito, o que as tornam fonte de infecção, fornecendo valiosa contribuição epidemiológica e informações aos programas de controle vetorial (Galardo, 2010; Brasil, 2020b).

Em áreas de garimpo no sul da Venezuela, Moreno *et al.* (2007) dissecaram o ovário de 2.091 espécimes pertencentes a *A. marajoara* e 1.201 de *A. darlingi* e as taxas de paridade foram de 81% e 60%, respectivamente. No Brasil, município de Anajás (Pará), 47,4% das

fêmeas de anofelinos já haviam realizado pelo menos uma oviposição, fator favorável à transmissão, pois indicam que as fêmeas estão sobrevivendo tempo suficiente para a realização do ciclo extrínseco do parasito, fato confirmado pela alta taxa de infectividade encontrada (Santos *et al.*, 2005).

1.5 Infecção natural por *Plasmodium* spp. em anofelinos

Para o controle e monitoramento da malária um dos principais parâmetros analisados é a detecção de espécies de plasmódios da malária humana nos anofelinos vetores (Li *et al*., 2001). Um dos primeiros estudos conduzidos na Amazônia brasileira realizou a dissecção de glândulas salivares em 1581 indivíduos pertencentes a *A. darlingi*, apenas 19 foram positivos para *Plasmodium* (Deane *et al*., 1948). Com o emprego de técnicas mais otimizadas para a época (radioimunoensaio - IRMA e imunoenzimática - ELISA), o número de espécies de *Anopheles* encontradas infectadas pelo *Plasmodium* aumentou na Amazônia brasileira, sendo listadas 14 espécies de anofelinos (Arruda *et al*., 1986; Neves, 2007).

No momento, para o diagnóstico da infecção natural de parasitos da malária em populações de anofelinos provenientes de áreas endêmicas, tem sido empregada a técnica de PCR (*Polymerase Chain Reaction*), pois trata-se de um método de diagnóstico de alta sensibilidade, especificidade, além do processamento de um grande número de espécimes com um ensaio automatizado (Singh *et al.*, 1999; Rocha *et al.*, 2008). Primeiramente, nessa técnica é realizada a extração do DNA do anofelino e posteriormente realizado um semi *nested-PCR* para a detecção de infecção plasmodial (Snounou *et al.*, 1993). Essa técnica tem sido amplamente considerada como padrão-ouro para detecção de espécies de plasmódios causadoras da malária humana (Rakotonirina *et al*., 2008). Na *nested-PCR*, o segmento genômico é amplificado especificamente para o gênero *Plasmodium* (1ª reação), e depois utilizado o primeiro produto da reação, para a amplificação da real sequência-alvo (2ª reação), específica para espécies de *Plasmodium* (Snounou *et al.*, 1993). Rocha *et al*. (2008) encontraram *Anopheles rondoni* infectado juntamente com *A. darlingi* e *A. albitarsis* s.l.

Conforme Coelho (2010), em estudo realizado na área de abrangência da Hidrelétrica de Curuá, no município de Santarém (Pará), foi aplicada a técnica de PCR para infectividade em 1.997 indivíduos, distribuídos em 317 *pools*; destes, nove foram positivos, sendo sete para *A. darlingi*, um *pool* para *A. albitarsis* s.l. e um para *A. nuneztovari* s.l. Por outro lado, Barbosa *et al*. (2016), utilizando a mesma técnica em anofelinos do Distrito do Coração (estado do Amapá), analisaram 397 *pools*, todos foram negativos para infecção por *Plasmodium*.

1.6 Habitats larvais – Caracterização e Parâmetros Limnológicos

Vários fatores bióticos e abióticos influenciam, de alguma forma, no desenvolvimento dos seres vivos, sendo o fator ecológico, o elemento capaz de interferir em pelo menos uma fase do ciclo de vida (Dantas, 2011). A densidade e o tamanho dos mosquitos que emergem dos criadouros sofrem influência da temperatura, precipitação, qualidade da água e evaporação (fatores abióticos), além da oferta alimentar, competição, predação e parasitismo (fatores bióticos), todos esses fatores influenciam nos criadouros (Barrera *et al*., 2006). Dantas (2011), ainda ressalta que cada habitat larval possui características estruturais (tamanho, forma, localização e sombreamento) e propriedades (matéria orgânica, comunidades microbianas e a entomofauna associada) que influenciam na biologia e no desenvolvimento dos mosquitos.

Comumente, os anofelinos não toleram águas poluídas, pois são criadouros caracterizados com baixo teor de matéria orgânica, constituídos por arbustos e locais com vegetação densa, podendo ser compostos por espaços sob raízes e troncos caídos, por grandes lagos ou lagoas, remansos de rios e córregos, represas artificiais, valas de irrigação, manguezais, pântanos e outros (Ferreira e Luz, 2003).

O avanço e aumento da densidade demográfica e implementação de vários empreendimentos urbanos, fez com que ocorresse a intensificação da pressão antrópica sobre os ambientes aquáticos (Cunha e Couto, 2002). Desse modo, é necessária a realização de pesquisas que possam diagnosticar os níveis dos impactos que atingem corpos da água e, consequentemente, a saúde humana (Cunha *et al*., 2003). A partir de informações técnico-científicas obtidas nessas pesquisas, é possível realizar o planejamento e gestão do recurso hídrico para que as atividades econômicas de desenvolvimento não conflitem com a capacidade de sustentabilidade ambiental (Cunha *et al*., 2003). A capacidade de suporte ambiental de uma coleção hídrica pode ser mensurada, através das grandezas físico-químicas que correspondem a um dos principais componentes dos parâmetros de qualidade da água (Cunha *et al*., 2003). Esses parâmetros da qualidade da água são estabelecidos por resoluções do Conselho Nacional do Meio Ambiente-CONAMA (Resolução nº. 357/2005 de 17 de março, e Resolução nº. 430/2011 de 13 de maio) que dispõe sobre a classificação dos corpos de água e diretrizes ambientais para o seu enquadramento, além de estabelecer as condições e padrões de lançamento de efluentes (Brasil, 2005; 2011). Entre os principais parâmetros aplicados para caracterizar físico-quimicamente as águas estão: cor, turbidez, temperatura, sólidos totais dissolvidos (TDS), condutividade elétrica, potencial hidrogeniônico (pH), oxigênio dissolvido (OD), fósforo e formas de nitrogênios (Richter e Azevedo Netto, 2003).

- Fatores Físicos:

Cor: normalmente está associada com a presença de sólidos dissolvidos e partículas coloidais, oriundos da decomposição de matéria orgânica, ferro e manganês, bem como de resíduos industriais e esgotos domésticos (Sperling, 2005).

Turbidez: está relacionada à presença de partículas em suspensão, sendo a profundidade de visibilidade correspondente com a faixa produtiva dos corpos hídricos. A presença dessas partículas impede que os raios luminosos penetrem nos corpos d'água, influenciando na fotossíntese e no crescimento das plantas aquáticas e do plâncton (Di Bernardo e Dantas, 2005).

Temperatura: a temperatura é um fator que influencia na maioria dos processos físicos, químicos e biológicos que ocorrem na água, sendo todos os organismos aquáticos adaptados para uma faixa ótima de temperatura, suportando oscilações (Richter e Azevedo Netto, 2003).

Sólidos Totais Dissolvidos (TDS): é um excelente parâmetro para analisar a qualidade ambiental, pois o excesso pode indicar contaminação orgânica recente por efluentes domésticos, industriais, ou por matéria sólida carregada pela erosão nas margens das coleções hídricas, comprometendo a vida aquática (Androeli *et al*., 2013).

Condutividade Elétrica: é a capacidade que a água possui de transmitir corrente elétrica, indicando a quantidade de sais existentes na coluna d'água e as possíveis modificações na sua composição, principalmente quanto à concentração mineral. Dessa forma. fornecendo dados sobre a concentração de poluentes (Dantas, 2011).

- Fatores Químicos:

Potencial Hidrogeniônico (pH): vários fatores interferem no pH, sendo considerados níveis ideais para a manutenção da vida aquática os valores entre 6,0 e 9,0. Valores fora dessa faixa são letais ou prejudiciais para a maioria dos organismos aquáticos (Cardoso, 2007).

Oxigênio Dissolvido (OD): este parâmetro indica a capacidade de manutenção da vida aquática, essencial para o metabolismo respiratório. A variação na concentração de OD ocorre sazonalmente, ou em ciclos de 24 h, em função da temperatura, quantidade de sais dissolvidos e atividade biológica, sendo assim um indicativo do nível de poluição da água (Sperling, 2005). Apesar dos imaturos de *Anopheles* respirarem oxigênio atmosférico, a distribuição do oxigênio no corpo d'água influencia na solubilidade dos nutrientes inorgânicos, essenciais para o desenvolvimento e sobrevivência das larvas de anofelinos (Dantas, 2011).

Fósforo: participa estruturalmente de várias moléculas fundamentais do metabolismo celular, como por exemplo, para o crescimento de bactérias, letais para as larvas durante o desenvolvimento (Smith e Prairie, 2004; Dantas, 2011). É um dos principais macronutrientes para os processos biológicos aquáticos e indispensável para o crescimento de algas, porém em níveis elevados causam a eutrofização do ambiente (Sperling, 2005).

Formas de nitrogênios: também correspondem a um macronutriente essencial para o crescimento das algas, sendo encontradas na água sob a forma molecular de amônia, nitrato e nitrito (Mota, 2012). Em baixas concentrações é um fator limitante para o desenvolvimento da vida aquática, enquanto em elevadas causam a eutrofização do ambiente (Sperling, 2005).

2 OBJETIVOS

2.1 Objetivo geral:

Elucidar a diversidade de *Anopheles* e os aspectos entomológicos envolvidos na transmissão da malária na Ilha de Santana, município de Santana, Estado do Amapá, Brasil.

2.2 Objetivos específicos:

- Analisar o padrão comportamental dos anofelinos adultos coletados, quanto as condições de endofília e exofília, a atividade horária hematofágica e aos percentuais de antropofília e zoofília;
- Identificar a composição da fauna de anofelinos adultos e imaturos;
- Estudar a bioecologia dos vetores;
- Determinar as espécies de vetores presentes e os tipos de criadouros existentes na área de estudo;
- Caracterizar a distribuição espacial das espécies coletadas nos estágios adulto e imaturo;
- Estudar a flutuação temporal dos anofelinos (adultos e imaturos) de acordo com variáveis climáticas, com ênfase nas espécies de importância médica;
- Caracterizar os habitats larvais preferenciais das espécies de *Anopheles* presentes na área de estudo;
- Inferir os parâmetros entomológicos envolvidos na transmissão da malária por meio da análise dos adultos (paridade e taxa de infectividade) e imaturos (densidade larval, índice de positividade e os índices de criação geral, absoluto e relativo);
- Caracterizar os parâmetros físico-químicos em amostras de água dos criadouros e as variáveis que influenciam na ocorrência e abundância de larvas de *Anopheles*;
- Identificar molecularmente os membros dos complexos de espécies coletados.

Capítulo I

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Blood-feeding behavior of *Anopheles* species (Diptera: Culicidae) in the district of Ilha de Santana, state of Amapá, eastern Brazilian Amazon

Ledayane Mayana Costa Barbosa[ab]; Vera Margarete Scarpassa[b]

[a]Laboratório de Arthropoda, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá. Rodovia Juscelino Kubitschek, km 02 - Jardim Marco Zero, CEP: 68903-419, Macapá, Amapá, Brasil. e-mail: barbosalmc@unifap.br

[b]Laboratório de Genética de Populações e Evolução de Mosquitos Vetores de Malária e Dengue, Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia. Av. André Araújo - Aleixo, CEP: 69.067-375, Manaus, Amazonas, Brasil. e-mail: vera@inpa.gov.br

Abstract

The present study aimed identifying the behavioral patterns of *Anopheles* species as well as to estimate the parity rate and natural infection analysis for *Plasmodium* species in the district of Ilha de Santana, state of Amapá, Brazil. The samples were obtained in four and 12-hours collections. In the intradomiciliary and peridomiciliary conditions and also in environments with the presence of animals from January/2017 to December/2018. The entomological parameters evaluated were human biting rate (HBR); Indexes of Anthropophily (I_A) and Zoophily (I_Z); Parity Rate (PR); Natural Infection Rate (NIR); Monthly and annual entomological inoculation rate (EIR). A total of 1,330 *Anopheles* specimens were collected, distributed in nine species. All captured species showed towards biting in outdoor environment. *Anopheles darlingi* was the most frequent species collected in indoor environment and the most anthropophilic (I_A = 0.39) compared with

the remaining species captured. It was also the unique species positive for *Plasmodium vivax*, had the highest biting activity throughout night, highest anthropophily degree and HBR in the first hours with a high rate of parous females. *Anopheles nuneztovari* s.l. was the most zoophilic species (I_Z = 0.65). These findings suggest that *A. darlingi* is the main malaria vector in the studied area. *Anopheles albitarsis* s.l. was the second species more anthropophilic (I_A = 0.31) and revealed a stable pattern with a biting activity peak after sunset, consequently this species may contribute with malaria transmission in area. Based on four-hour collections, the parity rate of *A. darlingi* ranged from 21.42 to 31.58, with the highest number of parous females (31.58) found in the third time interval (20 - 21h). While in 12-h collections, all *A. darlingi* females captured at the 01 - 02h time interval were parous (100.00). Another species with one of the highest parity rates was *A. albitarsis* s.l., varying between 30.00 and 100.00.

Keywords: Behavioral patterns; Entomological parameters; Malaria.

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Introduction

Malaria transmission in South America occurs in all Amazonian countries (Brazil, Bolivia, Peru, Ecuador, Colombia, Venezuela, Guyana, Suriname and French Guiana) (Ministério da Saúde do Brasil, 2020). In Brazil, approximately 68 species have already been reported, of which approximately 33 occur in the Brazilian Amazon, and 23

species have already been reported in the state of Amapá (Deane et al., 1948; Deane et al., 1971; Bergo et al., 2007; Galardo et al., 2015; Barbosa et al., 2016; WRBU, 2020). These species are distributed in five subgenera, with the ones with the greatest epidemiological importance in the subgenera *Kerteszia* Theobald, 1905 and *Nyssorhynchus* Blanchard, 1902 (Hiwat and Bretas, 2011). The latter includes the main species involved in the transmission of human malaria in the Brazilian Amazon region, including *A. darlingi* Root, 1926 and some members of the Albitarsis and Nuneztovari complexes (Hiwat and Bretas, 2011).

Anopheles darlingi is the main vector of *Plasmodium* species that cause the human malaria in the Brazilian Amazon, with broad behavioral plasticity (ranged from endophilic to exophilic and ranged from anthropophilic to zoophilic), increasing the complexity of the transmission dynamics of this disease (Santos et al., 2009). The degree of anthropophily is an essential condition for anopheline species to be considered an important vector of human malaria (Gouveia de Almeida, 2011) and, within the same taxon, the degree of anthropophily may vary according to the region (Forattini, 2002).

Variations in the behavioral patterns of anopheline species are influenced by external factors, such as ecological, environmental, and demographic. Human actions can also exert selective pressure on vector populations, benefiting them under new conditions (Kuwabara, 2008). For example, the deforestation and anthropization influence the local density of *A. darlingi*, which show high adaptive plasticity and degree of synanthropism (Vittor et al., 2006; Gomes et al., 2008). In addition, behavioral changes from endophily to exophily, resulted of the use of indoor residual spraying (IRS), are the selection of vector behavior and/or physiological resistance to insecticides (Tadei, 1987; Glunt et al., 2015; Ranson and Lissenden, 2016; Prussing et al., 2018).

Behavioral and ecological patterns of anopheline vectors can vary in space and time, including seasonal changes. Thus, local control strategies must be adequately planned according to the characteristics of the vectors (Ministério da Saúde do Brasil, 2019). Periodic studies evaluating behavioral patterns determined by the species composition of each area, density, interspecific interactions, biting behavior, intensity of human contact and female longevity are essential to monitor changes that may be associated with climate and environmental determinants (Barbosa et al., 2016; Ministério da Saúde do Brasil, 2019).

The behavior of the species involved in malaria transmission influences the local epidemiological pattern (Barbosa et al., 2016). The same population may show behavioral variations due to external changes, increasing the complexity of the disease transmission dynamics, as have been observed in *A. darlingi* populations (Voorham, 2002; Santos et al., 2009). Economic development, exploitation of forest products and migratory flow have driven the decrease in forest cover in the Brazilian Amazon. Deforestation is an important risk factor for the emergence of malaria transmission (Tadei et al., 1998; Chaves et al., 2018). These changes affect the development and proliferation of mosquitoes, in addition to socioeconomic factors associated with low Human Development Index (HDI) (migration, housing, population density, and income), as well as environmental (hydrology, climate, topography, and vegetation), biological (life cycle of vectors and pathogens and population immunity) and medical-sanitary components (health system effectiveness) (PAHO, 2009; Da Silva et al., 2010; Tadei et al., 2017). Despite advances in treatments with new drugs and attempts to develop vaccines, strategies to combat vectors, as well as land use and development planning are still lacking (Li et al., 2016; WHO, 2017; Chaves et al., 2018).

In 2019, an estimated 229 million malaria cases occurred worldwide and approximately 139 million people are at risk of contracting the disease in the Americas (WHO, 2020). In the Americas, Brazil, Colombia, and Venezuela combined account for 86% of the estimated cases of malaria. In Brazil, approximately 99.8% of them occur in the Amazonian region and the states with most malaria cases are: Acre, Amazonas, Amapá, Pará, Rondônia, and Roraima (SVS/SIVEP, 2020; WHO, 2020). In 2018 alone, ~194,512 malaria cases were reported in Brazil, of which 10,008 cases occurred in Amapá (SVS/SIVEP, 2019).

In the municipality of Santana, state of Amapá, 2,811 cases of malaria were reported in 2018, with an Annual Parasite Index (API) of 23.5. In the same year, 570 cases were reported in the district of Ilha de Santana, with an API of 241.3; thus, considered a high-risk area of malaria transmission (API ≥ 50) (SVS/SIVEP, 2019).

The present study aimed identifying the behavioral patterns of *Anopheles* species as well as to estimate the parity rate and natural infection rate for *Plasmodium* species in the district of Ilha de Santana, state of Amapá, an area where occur malaria transmission.

Materials and Methods

Specimens of *Anopheles* species were collected in the district of Ilha de Santana, municipality of Santana, state of Amapá, Brazil. This district is located on the banks of the Amazon River, between the geographical coordinates of 00º04’00’’and 00º06’00’’S and 51º08’00’’ and 51º12’30’’W, and comprises an area of 20.06 km^2 with an estimated population of 3,226 inhabitants (Madeira and Simões, 1972; Valente et al., 1998; IBGE, 2020). The municipality of Santana has an area of 1,541,224 km^2 and is located in the southern region of the state of Amapá, 25 km from the capital Macapá, bordering the

municipalities of Macapá, Mazagão and Porto Grande (IBGE, 2013). The main economic activity of the district of Ilha de Santana is based on the primary sector, with most residents working in agricultural activities (fruits and vegetables) and in the production of fruit pulps, especially of two important products of the açaí palm (*Euterpe oleracea* Mart): fruits and palm hearts (Valente et al., 1998).

Adult anophelines were collected from January 2017 to December 2018, covering the rainy and dry seasons. Active collections were carried out using the Protected Human Attraction Technique *(PHAT)* (Ministério da Saúde do Brasil, 2019), with a team formed by six experienced and properly trained collectors for this purpose. All team members used Personal Protective Equipment - PPE's, in comply with safety guidelines on the basic material required for sampling activities of the "Guide for Planning Anopheline Sampling by the Protected Human as Attraction Technique *(PHAT)* and Monitoring Health Risks of the Professional Collector" (Ministério da Saúde do Brasil, 2019). Anopheline specimens (females only) were collected with the aid of a flashlight, small insect collecting net, and a mouth aspirator, when trying to land on collectors, following Forattini et al. (1999).

The selection of sampling sites followed the criteria: (I) locations with the highest number of reports of malaria cases and (II) locations with the higher demographic density (IBGE, 2017). Sampling days were determined according to the lunar phases (Hosfall, 1943). Collections were carried out for four and 12 continuous hours and in habitats with the presence of animals (pigs, chickens, and/or cattle).

The collections were divided into three categories: the first one evaluated the levels of endophily and exophily. Collections were carried out concomitantly with one collector indoors (inside the dwelling - intradomiciliary) and another outdoors (within a

radius of up to ~30m away from the dwelling - peridomiciliary) from 18:00 to 22:00 hours in three different dwellings on the same day. The second category had the main goal of evaluating human biting rate and hourly biting activity in the peridomiciliary area from 18:00 to 6:00 hours. Three collectors participated in these collections, taking turns every two hours. Finally, the third category assessed parameters of anthropophily and zoophily in environments with pigs, chickens and/or cattle (extradomiciliary area) and in the peridomiciliary area, with two collectors, one collector in each area simultaneously from 18:00 to 22:00 hours. The three categories totaled 52 collection sites (Figure 1) (Table 1S, supplementary file).

All mosquitoes captured were placed in plastic cups and properly labelled with date, hour, site, and sampling method. Following Forattini (1962), mosquitoes were then transported in tightly closed isothermal boxes to the Laboratory of Arthropods of the Federal University of Amapá, where the morphological identification and dissection of ovaries were carried out to estimate parity rate. Anophelines were killed in a freezer at -20ºC and then identified under a stereo microscope Zeiss Stemi DV4, with the aid of dichotomous keys of Faran and Linthicum (1981), Consoli and Lourenço-de-Oliveira (1994), and Forattini (2002). The nomenclature used was proposed by Guimarães (1997). Damaged specimens that could not be identified were grouped as *Anopheles* species.

The collected individuals belonging to species complexes were subjected to molecular analysis to identify their members, using the DNA barcode region (Folmer region) of the *COI* gene of mitochondrial DNA (data no shown). From each species recognized as complex, a sample of 3% of the total captured was taken for analyzes. Other species were also sequenced to confirm their occurrence in the study area.

Endophily and Exophily

The four-hour collections carried out inside (indoors) the dwelling (intradomiciliary) and outdoors (peridomiciliary) were used to estimate the levels of endophily and exophily of the species. The variable level considered the number of mosquitoes (abundance) collected in each environment, analyzing the absolute and relative frequency.

Anthropophily and Zoophily

These parameters were evaluated using four-hour collections carried out specific for these analyses in the peridomiciliary area and in environments with the presence of animals (extradomiciliary area). The Index of Anthropophily (I_A) was determined based on anophelines attracted by the collector seeking a blood meal, while the Index of Zoophily (I_Z) was obtained based on anophelines searching to feed on the blood of animals or resting close to them. These parameters were then compared by separately computing the species collected near humans and animals, as described by Tadei et al. (1993).

These parameters were calculated as follows: I_A= TNSD/OTSD; where: TNSD = total number of specimens collected in dwellings per species divided by OTSD = overall total number of specimens of all species collected in dwellings. I_Z = TNSS/OTSS; where: TNSS = total number of specimens collected in sheds with animals per species divided by OTSS = overall total number of specimens of all species collected at the sheds.

Human biting rate (HBR)

Twelve-hour collections were carried out to examine the attraction of mosquitoes to humans. The index of attraction as mosquito/human/hour was calculated,

according to the formula: HBR = N/NC/CT, where: N = number of mosquitoes collected; NC = number of collectors; CT = collection time (Service, 1993).

Hourly biting activity

The time of highest activity and the hematophagic pattern of the species were analyzed using the data obtained from 12-hour collections.

Parity rate

All species and specimens captured during four and 12-hour collections were dissected. The abdomens were used for the dissection of ovaries for the analysis of parity rates, which were determined as nulliparous and parous based on the arrangement of tracheolar filaments, as described by Detinova (1962). After dissections, ovaries were examined under a Bel Photonics light microscope with 400x magnification. Parity rate (PR) was expressed according to the formula: PR = FP x 100/FD; where: FP = number of parous females and FD = number of dissected females.

Analysis of *Plasmodium* infection rate

Analysis of *Plasmodium* species infection rate was performed only for the most frequent species of mosquitoes captured during four and 12-hour collections. After identification, the heads and thoraces of females of *A. darlingi*, *A. albitarsis* s.l., *Anopheles braziliensis*, and *A. nuneztovari* s.l. were removed to estimate natural infection rates of *Plasmodium* species, which was detected by amplification of fragment of ribosomal DNA. The heads and thoraces were removed to refine the results and focus on the detection of sporozoites present in salivary glands, the infectious form of the parasite.

Head and thorax were placed in microtubes containing isopropanol, in pools by species, date, hour, site and collection method and preserved in freezer at -20ºC. The pools contained up to five heads and thoraces per species. The material was then

transported to the Laboratory of Population Genetics and Evolution of Malaria and Dengue Vectors of the Instituto Nacional de Pesquisas da Amazônia (INPA), in Manaus, where the samples were processed and analyzed.

DNA extraction was performed following the protocol described by Sambrook and Russell (2001). PCR reactions were performed following the protocol described by Snounou et al. (1993), which consisted of a semi-nested-PCR and two reactions. The second reaction, which identifies the *Plasmodium* species, was carried out only when the first reaction was positive for the *Plasmodium* genus. The primer sequences used for the detection of *Plasmodium* species as well as for *P. falciparum* Welch, 1897, *P. vivax* Grassi and Feletti, 1890, and *P. malariae* Laveran, 1881 followed Snounou et al. (1993). The PCR reactions were carried out in a VERIT thermocycler, Applied Biosystems. As a positive control, a sample confirmed for *P. vivax* was used, while the negative control consisted of DNA of *Anopheles konderi* Galvão and Damasceno, 1942 females reared in the insectary of the Laboratory of Population Genetics and Evolution of Malaria and Dengue Vectors of the INPA, first generation.

After amplification, PCR products were analyzed on a 1% agarose gel, where 8 µL of the amplified product and 2 µL of the GelRed dye were loaded onto the gel. After electrophoresis, the gel was examined under ultraviolet light and photo-documented with an imaging system coupled in the photodocumentary apparatus, Loccus Biotecnologia, model L-Pix Touch. The sizes of the obtained fragments were compared with the 100-bp ladder DNA for the first reaction and Low DNA Mass for the second reaction (Invitrogen®).

The detection rate of *Plasmodium* DNA was calculated using the minimum infection rate adapted from Forattini (2002), as MIR = N/I x 100; where: N = number of

positive pools to the infection test; I = total number of tested mosquitoes of a species. The Entomological Inoculation Rate (EIR), which indicates the number of infective bites that a person can receive per unit of time, was calculated as: EIR = HBR x MIR; where: HBR = Human biting rate and MIR = minimum infection rate. For the monthly analysis of EIR, the product was multiplied by 31 (days) and for the annual analysis, the product was multiplied by 365 (days) (Williams and Pinto, 2012).

Data analysis

Means were used to compare endophily/exophily and anthropophily/zoophily with the Student's *t* test and the nonparametric substitute Mann-Whitney test, according to the data distribution.

The statistical analysis was based on the Generalized Linear Model (GLM) with the Poisson distribution. Poisson models were used to assess whether the target variable *Anopheles* abundance was influenced by the environment (intra and peridomiciliary), hematophagic activity time, and other species. Abundance was used as a response variable and environment (intra and peridomiciliary), species activity time, and species as predictor variable. GLM was also carried out to assess whether abundance was influenced by feeding preference (anthropophily and zoophily), species activity time, and other species. Considering abundance as a response variable and feeding preference (anthropophily and zoophily), species activity time and species as predictor variable. These analyzes were performed using the software R (2021). The Kruskal-Wallis test was carried out to analyze HBR, hourly biting activity, and parity rate.

The most abundant species, especially the most frequent species indoor and outdoor environments were plotted in graphs with the number of specimens, by collection time intervals, combined with parity rate and number of malaria cases (data obtained from

the Computerized Health System - Epidemiological Surveillance – SIVEP/MS). To test variable associations, Spearman correlations were used. Descriptive statistics were obtained with the software BioEstat version 5.0 (Ayres et al., 2007). The significance level of $\alpha = 0.05$ was used.

Ethics

This study was approved by the Research Ethics Committee of the Federal University of Amapá, registered under #78912617.9.0000.0003. The collection and transport of the target specimens was authorized by Brazilian Environmental Institute (IBAMA) through the Biodiversity Information and Authorization System (SISBIO) under #52442-1.

Results

A total of 1,330 *Anopheles* specimens were collected in the study area, distributed in nine species and two subgenera: *Nyssorhynchus* (six species) and *Anopheles* (three species). The species *A konderi* and *Anopheles triannulatus* were identified by molecular methods. For specimens belonging to the Albitarsis and Nuneztovari complexes, sequences of high quality could not be obtained, making comparisons with those deposited in GenBank impossible; therefore, in this study, the specimens were denominated as *A. albitarsis* s.l. and *A. nuneztovari* s.l., respectively. The sequenced specimens of *A. braziliensis*, *A. darlingi*, and *Anopheles intermedius* (Peryassú, 1908) confirmed the occurrence of these species in the study area. Of the total, 276 (20.75%) were captured in four-hour collections (intradomiciliary and peridomiciliary), 169 (12.71%) in the 12-hour collections (only peridomiciliary), and 885 (66.54%) in four-hour collections (humans and animals), totaling 272 hours of sampling effort (Table I).

Considering all sampling methods, *A. nuneztovari* s.l. was the most common captured species (45.26%) in the area and with 83.53% of the specimens collected when evaluating anthropophilic and zoophilic indexes. The second most abundant species was *A. darlingi* (19.10%), followed by *A. albitarsis* s.l. (18.57%), and *A. braziliensis* (10.00%) (Table I).

Behavioral patterns

- Endophily and Exophily

All species were captured more frequently in outdoor environment (76.45%). *Anopheles mattogrossensis* Lutz and Neiva, 1911, *A. konderi*, *Anopheles peryassui* Dyar and Knab, 1908 and *A. triannulatus* were collected in low frequencies (lower than 5%) (Table II).

The levels of endophily and exophily observed for *Anopheles* species during the sampling period did not reveal significant differences between environments (Mann-Whitney U test = 24; z = -1.42; p = 0.16), although a predominance for the outdoor environment has been observed. Only one exception was recorded, represented by only one individual (*A. darlingi*), which was collected in the indoor environment in May/2017. In the months of January and July 2017, no individuals were collected in the indoor environment.

Figure 2 shows the levels of endophily and exophily for *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi*, and *A. nuneztovari* s.l. Despite of predominance in outdoor environment, *A. darlingi* was the species that had the highest number of specimens collected in the indoor environment. *Anopheles albitarsis* s.l. was also captured in indoor environment in March/2017, July/2018, September/2018, and November/2018. The behavioral patterns of *A. braziliensis* and *A. nuneztovari* s.l. varied considerably during

the sampling period. However, a significant difference between the two environments was observed only for the abundance of *A. albitarsis* s.l. throughout the sampling period (Mann-Whitney U test = 31.5; z = -2.44; $p = 0.01$).

Regarding the collection time interval for the more frequent species collected, *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi* and *A. nuneztovari* s.l. showed greater abundance in the second time interval (Figure 3). A significant difference between indoor and outdoor environments was found for *A. albitarsis* s.l. and *A. braziliensis* when analyzing by time interval, indicating that the number of mosquitoes biting outdoors is significantly higher than that of those biting indoors (Student's *t* test [3] = -3.44; $p = 0.01$, Student's *t* test [3] = -2.49; $p = 0.04$; respectively).

GLM revealed that species abundance varied between intra and peridomiciliary environments, indicating the exophilic preference of *Anopheles* species, reflected in a very significant and positive relationship. Regarding abundance during collection times, a significant and negative relationship was observed, showing that anopheline activity was highest in the second collecting period. Another aspect analyzed was the general abundance of *Anopheles* species in relation to the abundance of the four most frequently collected species. A statistically significant and positive relationship was also found, showing that these species (*A. albitarsi*s s.l., *A. braziliensis*, *A. darlingi*, and *A. nuneztovari* s.l.) occur in greater abundance in the District of Ilha de Santana (Table III).

- Anthropophily and Zoophily Indexes

Regarding anthropophilic and zoophilic indexes, the 885 mosquitoes collected were distributed in nine species: *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi*, *A. intermedius*, *A. konderi*, *A. mattogrossensis*, *A. nuneztovari* s.l., *A. peryassui* e *A. triannulatus*. Highest abundance was observed in February/2017, with 346 (39.10%)

individuals, followed by February/2018, with 260 (29.38%) specimens (mainly *A. nuneztovari* s.l. and *A. albitarsis* s.l.). The lowest abundance was observed in November/2017, with four (0.45%) individuals (*A. albitarsis* s.l. and *A. darlingi*). In all months, specimens were predominantly collected displaying zoophilic behavior, with the exception of November/2017 that showed two specimens (*A. albitarsis* s.l. and *A. darlingi*) collected in anthropophilic conditions and two in zoophilic conditions (*A. albitarsis* s.l.). In May/2018, 28 specimens were collected near dwellings and only one specimen (*A. intermedius*) was captured in the environment with animals. All species were more frequently collected displaying zoophilic behavior, except for *A. mattogrossensis* and *A. peryassui* with one (0.11%) and three (0.34%) individuals collected, respectively, displaying anthropophilic tendencies. *Anopheles nuneztovari* s.l. was the most abundant species, with 502 (56.72%) specimens collected predominantly in the environment with animals. Of these, only seven (1.39%) were captured near dwellings. Two peaks were observed for this species throughout the collection period, February/2017 and February/2018, with 239 (47.61%) and 231 (46.01%) specimens collected, respectively (Figure 4). The second most abundant species was *A. albitarsis* s.l., with 176 (19.89%) and the highest abundance was observed in February/2017 with 82 (46.59%) individuals (Figure 4). *Anopheles darlingi* was the third most frequent species with 110 (12.43%) individuals collected and showed a peak in August/2018, with 60 (55.55%) specimens captured. Of these, 32 (53.33%) were captured near dwellings and 28 (46.67%) in the environment containing animals. For this species, a simultaneous variation was observed between anthropophilic and zoophilic indexes throughout the collection period. *Anopheles braziliensis* was the fourth the most abundant species, with 66 (7.46%) specimens and showed a peak of anthropophilic behavior in May/2018,

represented by 17 individuals, and a peak of zoophilic behavior in November/2018, with 41 specimens collected (Figure 4). No significant differences in abundance between anthropophilic and zoophilic behaviors were found for *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi*, and *A. nuneztovari* s.l.

Of the 885 mosquitoes collected, 119 (13.45%) individuals displayed anthropophilic behavior and 766 (86.55%) specimens showed zoophilic behavior. In environment containing animals, with the exception of the first-time interval, a high abundance was observed in all other times. The species with the highest anthropophilic index was *A. darlingi* (I_A = 0.39), followed by *A. albitarsis* s.l. (I_A = 0.31), and *A. braziliensis* (I_A = 0.19), whereas *A. nuneztovari* s.l. (Iz = 0.65) had the highest zoophilic index (Table II; Figure 5). *Anopheles intermedius*, *A. konderi*, *A. mattogrossesnsis*, *A. peryassui*, and *A. triannulatus* had anthropophilic and zoophilic indexes equal or lower that 0.03%. No significant differences between the anthropophilic and zoophilic indices obtained per species were observed (Mann-Whitney U test = 36.5; z = -0.31; p = 0.75).

When taking into account species distribution per time interval, the number of mosquitoes collected displaying zoophylic behavior was much higher than those captured displaying anthropophilic behavior (Figure 6). However, only *A. albitarsis* s.l. showed significant differences between the anthropophilic and zoophilic behaviors per time interval (Student's t test [3] = -2.67; p = 0.03).

GLM was also used to determine feeding preferences (anthropophily/zoophily), time of highest hematophagic activity considering feeding preference, and ratio of general abundance to abundance of the four species collected in greater frequency. Although no significant differences were found between anthropophilic and zoophilic indices (mentioned above), GLM revealed a trend regarding feeding preference, which was as a

very significant factor in the abundance of species, indicating preferentially a zoophilic behavior. Another determining factor was collection time, which showed a very significant relationship, confirming the highest zoophilic activity during the second collecting period. Finally, when analyzing the relationship between general abundance and the abundance of the four most frequently collected species, a significantly strong relationship was observed, demonstrating the predominance of the four species collected at the highest densities in the study area (Table III).

- Human biting rate (HBR)

The highest index of attraction as mosquito/human/hour was observed for *A. nuneztovari* s.l. with 3.00 in the interval 19 – 20h. *Anopheles albitarsis* s.l. had the second highest HBR, ranging from 0.08 to 0.83 at the time intervals: 01 – 02h, 04 – 05h, and 18 – 19h. The third species with the highest index was *A. darlingi* with 0.75, which was collected in almost all time intervals, except in the interval of 20 – 21h (Figure 7). The remaining species showed HBR ranging from 0.00 to 0.33.

The analysis of HBR for 12-h collections revealed a significant difference between *A. darlingi* and the species *A. intermedius*, *A. konderi*, *A. mattogrossensis*, *A. peryassui*, and *A. triannulatus* (Kruskal-Wallis test [8] = 33.02; $p < 0.05$). Significant differences in HBR were found between the time intervals: 18 – 00h, 19 – 23h, 19 – 00h, 19 – 01h, 19 – 02h, 19 – 03h, 19 – 04h, and 19 – 05h (Kruskal-Wallis test [11] = 15.16; $p < 0.05$).

- Hourly biting activity

Considering all species captured during 12-h collections, a bimodal peak between the time intervals of 21 – 22h and 02 – 03h was observed for *A. mattogrossensis*, while *A. konderi* was collected only in the first time interval, consequently showed a

unimodal peak. It should be pointed out because these species were captured in low densities. *Anopheles braziliensis* and *A. triannulatus* had multimodal peaks, although the latter was also found in low densities.

Two well-defined peaks of feeding activity (18 – 19h and 21 – 22h) were observed for *A. darlingi*, which was not collected in only one time interval (20 – 21h). On the other hand, a unimodal pattern with a peak early in the evening (19 – 20h) was found for *A. albitarsis* s.l., which was absent in the 22 – 23h; 23 – 00h; 00 – 01h; 03 – 04h; 05 – 06h time intervals.

Regarding the hourly biting activity of the main captured species, a significant difference was found between *A. darlingi* and the species *A. intermedius, A. konderi, A. mattogrossensis, A. peryassui* and *A. triannulatus*; between *A. albitarsis* s.l. and *A. konderi*; and between *A. mattogrossensis* and *A. peryassui* (Kruskal-Wallis test [11] = 18.79; $p < 0.05$). Activity was clearly higher during the 19 and 20h time interval, with a significant difference in biting activity between 18 and 00h, 19 and 23h, 19 and 00h, 19 and 01h, 19 and 02h, 19 and 03h, 19 and 04h, and 19 and 05h (Kruskal-Wallis test [8] = 40.92; $p < 0.05$).

- Parity rate

Based on four-hour collections, the parity rate of *A. darlingi* ranged from 21.42 to 31.58, with the highest number of parous females (31.58) found in the third time interval (20 - 21h). The parity rates for *A. albitarsis* s.l. (42.86) and *A. braziliensis* (50.00) were highest in the first time interval (18 - 19 h). All specimens of *A. konderi* and *A. mattogrossensis* captured were nulliparous. Parous females of *A. darlingi, A. albitarsis* s.l., *A. braziliensis,* and *A. intermedius* were found at all time intervals.

No significant differences in parity rates were found among collection time intervals. However, a significant difference in parity rates was found between *A. darlingi* and *A. intermedius* and between *A. nuneztovari* s.l. and *A. intermedius* (Kruskal-Wallis test [6] = 9.65; $p < 0.05$).

In 12-h collections, all *A. darlingi* females captured at the 01 - 02h time interval were parous (100.00), with the parity rate ranging from 25.00 to 100.00. Another species with one of the highest parity rates was *A. albitarsis* s.l., varying between 30.00 and 100.00, with all parous females at the 04 – 05h time interval. Analyzing parity rates by time, a significant difference was found between the intervals 18 and 00h, 19 and 20h, 19 and 22h, 19 and 00h, 19 and 02h and 19 and 03h (Kruskal-Wallis test [11] = 11.27; $p < 0.05$). When analyzing parity rates by species, a significant difference was observed between *A. albitarsis* s.l. and *A. mattogrossensis, A. albitarsis* s.l. and *A. konderi, A. darlingi* and *A. intermedius, A. darlingi* and *A. mattogrossensis, A. darlingi* and *A. konderi,* and *A. darlingi* and *A. triannulatus* (Kruskal-Wallis test [5] = 16.08; $p < 0.05$).

In July and September 2017 there was a rise in the number of malaria cases accompanying the increase in anopheline density. For *A. darlingi,* the increase in parity rate was simultaneous with the increase in density of this species, with an overlap in variables. Also, when analyzing the increase in density and the increase in the number of malaria cases, a synchrony during the two malaria peaks also occurred (September/2017 and November/2018) (Figure 8). The increase in density of *A. albitarsis* s.l. was followed by an increase in the number of cases malaria, with peaks in parity rate along with density (Figure 8). The same was observed for *A. braziliensis* regarding the increase in density with the number of malaria cases (Figure 8). For *A. nuneztovari* s.l., a simultaneous variation between density and parity was observed; however, when analyzing density and

number of malaria cases, the increase in the number of individuals of this species clearly occurred only after the increase in the number of malaria cases (Figure 8). The density and parity rate of *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi* and *A. nuneztovari* s.l. showed a strong, positive and significant correlation. However, no correlation was found between density and number of malaria cases or between parity rate and the number of malaria cases. Despite the already mentioned synchronism observed for *A. darlingi* between density and number of malaria cases.

- Minimum infection Rate (MIR) and Entomological inoculation rate (EIR)

In this study, 438 pools were analyzed for *Plasmodium* species using the PCR technique, distributed as follow: 121 for *A. darlingi*, 95 for *A. albitarsis* s.l., 54 for *A. braziliensis*, 163 for *A. nuneztovari* s.l. and five for *Anopheles* species. Of these, only one pool was positive for *P. vivax*, which contained one specimen of *A. darlingi*. This specimen was captured during 12-h collections, between 18 and 19 hours and in May/2018, when only ten mosquitoes were collected, of which five were *A. albitarsis* s.l. and five were *A. darlingi*. However, the specimens of *A. darlingi* were captured at different times. The minimum infection rate was 0.83% for *P. vivax*, while the monthly EIR was 0.11 infectious bites/human/month and the annual EIR was 1.27 infectious bites/human/year.

Discussion

In this study, a total of nine species was captured and the findings revealed that, of the four most abundant species, two (*A. darlingi* and *A. albitarsis* s.l.) have been appointed as the most important malaria vectors in the state of Amapá (Póvoa et al., 2001; Galardo et al., 2007; Barbosa et al., 2016).

The collected species were recorded in all sampling methods, revealing their behavioral plasticity. Although most individuals were collected outdoors, the main species involved in the transmission of malaria in the state of Amapá (*A. darlingi* and *A. albitarsis* s.l,) were also collected indoor environment and had the highest anthropophily index (I_A = 0.39; 0.31, respectively). *Anopheles nuneztovari* s.l. was captured in highest density in this study, but it was the most zoophilic species (I_Z = 0.65). Although *A. nuneztovari* s.l. is considered an important malaria vector in Colombia and Venezuela (Rubio-Palis et al., 1992; Schoeler et al., 2003; Turell et al., 2008; Sinka et al., 2010; Naranjo-Díaz et al., 2016), in Brazil this species is considered a secondary vector in some regions, in spite to be found frequently infected with *Plasmodium* species (Santos et al., 2005; Galardo et al., 2007; Rezende et al., 2009). However, some studies carried out in the state of Amapá indicate that *A. nuneztovari* s.l. may contribute to malaria transmission, especially when in high densities (Galardo et al., 2007; Barbosa et al., 2016). In fact, in the Brazilian Amazon this species may consist of two or more species, as was demonstrated by Scarpassa et al. (2016).

The tools for the control of malaria vectors recommended by the World Health Organization (WHO) are long lasting insecticide treated nets (LLINs) and indoor residual spraying (IRS). However, these methodologies mainly target endophilic anophelines (Prussing et al., 2019). In this study, these control measures can become ineffective against the malaria vectors in the district of Ilha de Santana, due behavior to be predominantly outdoors. An example of unsuccessful IRS was reported in Peru, where Prussing et al. (2018) did not observe a consistent effect of spraying on the abundance of *A. darlingi*. In this case, this method was insufficient to eliminate malaria, and additional strategies were needed (Williams et al., 2018; Prussing et al., 2019).

The GML revealed a significant effect of the variable environment (indoors and outdoors) on abundance of *Anopheles* species. *Anopheles albitarsis* s.l. had the second highest index of attraction as mosquito/human/hour. Since the first studies carried out in the state of Amapá by Deane et al. (1948), *A. albitarsis* s.l. has been considered a possible secondary vector of malaria, and its epidemiological importance has been supported by Póvoa et al. (2001), Conn et al. (2002), Galardo et al. (2007) and Barbosa et al. (2016), either as primary vector at times or helping the maintenance of the disease in areas of transmission. In this study, *A. albitarsis* s.l. was found at lower densities throughout the sampling period, and may be helping the maintenance of malaria in the region and playing a role as a secondary vector of the disease.

The greater abundance of anopheline vectors collected outdoor environment allows the assessment of the level of risk of malaria transmission, given the common habit of residents be outside their residences at dusk, when anopheline activity was highest. In this study, *A. darlingi* exhibited anthropophilic and crepuscular behaviors (dawn and dusk). When analyzing the entire sampling period, however, zoophilic behavior was also observed for this species during the collections carried out in environments with animals, indicating its hematophagic plasticity. When analyzing anthropophilic and zoophilic behaviors by time interval, the anthropophily index was highest in the two species, *A. darlingi* and *A. albitarsis* s.l., demonstrating their blood-feeding preferences and epidemiological importance (Barbosa et al., 2016). Also, in 12-hour collections, the number of parous females of *A. darlingi* and *A. albitarsis* s.l. was highest in the first time intervals, confirming that these hours are when the risk for malaria transmission is highest. However, parity rate for both species peaked in the last hours of the night.

Similar results to those observed in the present study were also reported in the state of Maranhão for *A. albitarsis* s.l., *A. darlingi*, and *A. nuneztovari* s.l., regarding HBR and with peaks occurring in the first time intervals (Barros et al., 2020). A second peak in the third time interval (21 – 22 hours) was observed for *A. darlingi*. In the present study, this species had the third highest HBR and was found in almost all collection times, indicating its activity throughout the night (time plasticity). *Anopheles albitarsis* s.l. and *A. braziliensis* were captured predominantly in outdoor environment, however, they displayed anthropophilic behavior (I_A = 0.31; 0.19, respectively). These species were less anthropophilic than *A. darlingi* (I_A = 0.39). *Anopheles albitarsis* s.l. has been implicated as local vector of malaria in the states of Amapá (Conn et al., 2002; Galardo et al., 2007; Barbosa et al., 2016) and Roraima (Silva-Vasconcelos et al., 2002). Considering the findings obtained with *A. braziliensis*, further studies are needed to clarify its role in malaria transmission in this area and in other areas of Amapá.

Anopheles intermedius, *A. konderi*, *A. mattogrossensis*, *A. peryassui*, and *A. triannulatus* were found in low densities in all sampling methods, with significant differences in HBR rates compared with *A. darlingi*, supporting that these species are not epidemiologically important in malaria transmission in the studied area. However, some studies have reported *A. triannulatus* naturally infected with *Plasmodium* species in Venezuela and Colombia (Moreno et al., 2009; Rosero et al., 2013), and in some states of the Brazilian Amazon (Galardo et al., 2007; Moreno et al., 2013). Despite the wild and zoophilic behavior of these species, *A. triannulatus* can play a role as secondary vector when in high densities, behaving as an opportunistic species, depending on host availability and abundance (Galardo et al., 2007; Rosero et al., 2013). These variations in behavior may be associated with the existence of a cryptic species complex, consisting of

at least three species (*Anopheles triannulatus* s.s. Neiva and Pinto, 1922; *Anopheles halophylus* Silva-do-Nascimento and Lourenço-de-Oliveira, 2002; and *Anopheles triannulatus* C Silva-do-Nascimento et al., 2006).

Although *A. nuneztovari* s.l. was found indoors, this species was abundant in environments with animals, where it displayed a zoophilic behavior throughout the sample period, confirming its feeding preference in the study area. Members of the Nuneztovari complex are widely distributed from eastern Panama to northern South America and throughout the Amazon basin, in Bolivia, Brazil, Colombia, Ecuador, Guyana, Peru, and Venezuela (Sinka et al., 2010). This complex comprises four species: *Anopheles nuneztovari* s.s. Gabaldón, 1940; *Anopheles goeldii* Rozeboom and Gabaldón, 1941; *Anopheles dunhami* Causey, 1945; and *Anopheles nuneztovari* cytotype A (Sant'Ana et al., 2015; Dos Santos et al., 2019), with variations in behavior, vector capacity, and involvement in malaria transmission. In the Brazilian Amazon, *A. nuneztovari* s.l. may be a local vector, displaying mainly exophilic and zoophilic behaviors (Galardo et al., 2007; Barbosa et al., 2016). However, in Colombia and Venezuela, *A. nuneztovari* s.s. is endophilic, exophilic and anthropophilic (Moreno et al., 2007; Gutiérrez et al., 2009; Naranjo-Díaz et al., 2013; Naranjo-Díaz et al., 2016).

Despite a higher abundance of zoophilic individuals collected in the months of February, May and August 2017, *A. darlingi* was collected displaying both anthropophilic and zoophilic behaviors in nearly equal numbers during the entire sampling period, but with the index of anthropophily slightly higher. Regarding biting activity, although *A. darlingi* was active in almost throughout night, a bimodal pattern was observed early in the evening, between 18 and 19 hours and between 21and 22 hours.

Anopheles darlingi does not have well-defined standards of biting activity (Gil et al., 2015; Lainhart et al., 2015; Moreno et al., 2015; Tadei et al., 2017; Saavedra et al., 2019), likely due to environmental variables and very high levels of intra-population genetic variation. *Anopheles albitarsis* s.l. was more active early in the evening, with a lower peak at 02 hours. This unimodal pattern was also reported in other location from Amapá (Voorham, 2002) and in the state of Roraima (Póvoa et al., 2006), Brazil, and in western Venezuela (Rubio-Palis and Curtis, 1992).

During four-hour collections, parity rates of *A. darlingi* and *A. albitarsis* s.l. were high, with occurrence of parous females at all time intervals, indicating a greater risk of malaria transmission. The increase in the number of malaria cases coincided with the increase in *A. darlingi* density, as well as with in parity. These results, combined with a positive test for *P. vivax*, clearly indicate the involvement of this species in the malaria transmission in the district of Ilha de Santana, and confirm its role as the main malaria vector in the Amazon region. Our findings about the behavior of *A. darlingi* in the state of Amapá is in agreement with the reported by Deane et al. (1948), Lourenço-de-Oliveira et al. (1989), Klein and Lima (1990), Quintero et al. (1996), Tadei et al. (1998), Moutinho et al. (2011) and Barbosa et al. (2016).

Anopheles darlingi was positive with *P. vivax* in the first time interval (18 – 19h), confirming the time of greatest risk of malaria infection by residents. Despite the single positive pool, the infection rate was similar to those found in Cahuide and Santa Emilia, in Peru (Prussing et al., 2018). In the Americas, the highest prevalence of malaria is caused by *P. vivax*, representing 75% of cases of infections, a milder and rarely lethal form (WHO, 2019). However, this type of malaria is responsible for most cases of relapse,

as it develops latent forms in the liver cells (hypnozoites), and can remain inactive for years (White et al., 2014).

In this study, although the rate of infection with *Plasmodium* species was low, the district of Ilha de Santana is an area susceptible to malaria outbreaks, because consecutive epidemic cycles can occur even with low parasitemia (Klein et al., 1991; Alves et al., 2005). The main species responsible for the transmission of malaria were identified in the study area in high densities compared to other species. This is an important public health issue, since the migratory flow of people to the district of Ilha de Santana from other malarial areas of the Amazon may promote the local spread of the malaria parasite, triggering the disease (Barbosa et al., 2014).

Anopheles darlingi is the main vector of malaria in the Brazilian Amazon and one of the most anthropophilic anophelines, and its behavior is very heterogeneous. The district of Ilha de Santana has favorable characteristics for the development of this species, with areas of preserved vegetation (forest area, savanna, mangrove) and streams. In addition, human activity has increased in this district, as a result of deforestation for the development of agricultural activities and the construction of fish tanks, as well as intense migratory flow. These factors can favor the increase of the density of *A. darlingi* and contribute to the continuous transmission of malaria.

When analyzing the correlation between density and parity of *A. darlingi*, *A. albitarsis* s.l., *A. braziliensis*, and *A. nuneztovari* s.l., a strong correlation was observed, indicating that the higher the abundance is associated with the higher the number of parous females. In the months when the density of these species increases, *Plasmodium* circulation increases, as well as the chances of outbreaks.

In Venezuela, in riverine villages located in Bolívar, of 2,707 mosquitoes analyzed by ELISA, only two pools of *A. darlingi* were positive out of a total of 1,118 specimens analyzed (Rubio-Palis et al., 2013). In a study carried out in Colombia, the sporozoite rate obtained was 0.13%, lower than our findings, and only one positive individual of *A. darlingi* was found, also for *P. vivax* (Jiménez et al., 2014). Overall, infection rates in anophelines are very low, varying between 0.1 and 3.7% (Tucker Lima et al., 2017), supporting our findings.

In the present study, some factors may have influenced the low density of anophelines throughout the collections, such as the habit of some residents to light fires in front of homes to repel mosquitoes and the use of pesticides in plantations, a common practice in the rural area of Ilha de Santana. Indoor residual spraying in September/2017 during three consecutive days and in March and May/2018 by the Health and Surveillance Coordination, likely contributed to the low abundance of anophelines species in the studied area. Another factor that might have influenced this reduction was the intense fires, a very common practice between September and November, which are the months warmest and driest of the year in region.

Conclusion

Anopheles darlingi was the most abundant species in four-hour collections, the most frequent anopheline indoors, the most anthropophilic, and the only species positive for *P. vivax*, confirming its epidemiological importance and its involvement in the transmission of malaria in the district of Ilha de Santana. *Anopheles darlingi* also had the highest hourly biting activity and HBR in the first hours with a high parity rate, confirming the early evening as period with the highest activity of this species and

coinciding with time when residents are most exposed. *Anopheles albitarsis* s.l. was also the second species more anthropophilic, consequently it may play an important role in the transmission of malaria in this district, harmoniously coexisting with *A. darlingi*. The behavior of *A. braziliensis* was a similar to that of *A. albitarsis* s.l., being found predominantly outdoors with some anthropophilic tendencies. An increase in density was observed for this species, with peaks that preceded a rise in malaria cases. Therefore, the role of *A. braziliensis* as malaria vector in the study area needs to be investigated. Although *A. nuneztovari* s.l. had highest density in this study, it was collected predominantly in outdoor environment and displayed a zoophilic behavior, with peaks after the increase in the number of malaria cases. Taken together, these findings suggest that this species is not involved in malaria transmission in the district of Ilha de Santana.

Finally, although we recognized the importance of current control methods applied in the district of Ilha de Santana, we recommend complementary control strategies tailored to local conditions, given the inherent characteristics of *A. darlingi*, its high adaptive capacity in anthropic environments as well as its behavioral plasticity (predominantly outdoors) and heterogeneity, in order to meet the goals established by WHO in the control and eradication of malaria.

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Table 1S, supplementary file – Details of the methodology during the study, according to the collection categories, district of Ilha de Santana, municipality of Santana, state of Amapá.

Collection design			
Period	24 months		
Collections	Bimonthly	Quarterly	Quarterly
Collection time (hourly)	Four-hour collections (from 18:00 to 22:00).	12-hour collections (from 18:00 to 6:00).	Four-hour collections (from 18:00 to 22:00).
Total collections	12	8	8
Number of sampling points (dwelling) / collection	3	1	1
Total sample points	36	8	8
Parameters analyzed	- Endophily and Exophily; - Parity; - Detection and identification of *Plasmodium* spp.	- Human biting rate (HBR); - Hourly biting activity; - Parity; - Detection and identification of *Plasmodium* spp.	- Anthropophily and Zoophily; - Detection and identification of *Plasmodium* spp.
Number of collectors per collection	6	3	2

Collector location	- 1 collector indoors (inside the dwelling - intradomiciliary); - 1 collector outdoors (within a radius of up to 30m from the dwelling - peridomiciliary) (simultaneously).	- 1 collector in the domiciliary area (collectors took turns every two hours).	- 1 collector in the domiciliary area; - 1 collector in environments with pigs, chickens and/or cattle - extradomiciliary (simultaneously).
Collection methods	- Protected Human Attraction Technique (PHAT).	- Protected Human Attraction Technique (PHAT).	- Protected Human Attraction Technique (PHAT) (domiciliary area); - Active search for anophelines feeding on animals or resting close to them (environments with the presence of animals).

Table I – Species of *Anopheles* and absolute and relative frequency, according to the collection categories in the district of Ilha de Santana, municipality of Santana, state of Amapá (Kruskal-Wallis test [13] = 1.02; $p > 0.05$).

Species	4-h collections (Intra./Peri.)		12-h collections (Peri.)		4-h collections (Humans/Animals)		Total (%)
	nº	**%**	**nº**	**%**	**nº**	**%**	
Anopheles (*Ny.*) *albitarsis* s.l.	42	**17.00**	29	**11.74**	176	**71.26**	**247 (18.57)**
Anopheles (*Ny.*) *braziliensis*	47	**35.34**	20	**15.04**	66	**49.62**	**133 (10.00)**
Anopheles (*Ny.*) *darlingi*	100	**39.37**	44	**17.32**	110	**43.31**	**254 (19.10)**
Anopheles (*An.*) *intermedius*	21	**61.76**	7	**20.59**	6	**17.65**	**34 (2.56)**
Anopheles (*Ny.*) *konderi*	2	**40.00**	1	**20.00**	2	**40.00**	**5 (0.37)**
Anopheles (*An.*) *mattogrossensis*	1	**25.00**	2	**50.00**	1	**25.00**	**4 (0.30)**
Anopheles (*Ny.*) *nuneztovari* s.l.	44	**7.31**	56	**9.32**	502	**83.53**	**602 (45.26)**
Anopheles (*An.*) *peryassui*	6	**60.00**	1	**10.00**	3	**30.00**	**10 (0.75)**
Anopheles (*Ny.*) *triannulatus*	13	**36.11**	9	**25.00**	14	**38.89**	**36 (2.71)**
Anopheles spp.	-	**-**	-	-	5	**100.00**	**5 (0.38)**
Total (%)	**276**	**20.75**	**169**	**12.71**	**885**	**66.54**	**1,330 (100.00)**

Table II – *Anopheles* species collected indoors (endophily) and outdoors (exophily), and peridomiciliary area (anthropophily) and in environments with the presence of animals (extradomiciliary - zoophily) in four-hour collections, in the district of Ilha de Santana, municipality of Santana, state of Amapá (Mann-Whitney U test = 24; z = -1.42; $p = 0.16$).

Species	Endophily and Exophily			Anthropophily and Zoophily				
	Intra.	Perid.		Human	Animals			
	Individuals number (%)	Individuals number (%)	Total (%)	Individuals number (%)	Individuals number (%)	Total (%)	I_A	I_Z
A. (*Ny.*) *albitarsis* s.l.	6 (14.29)	36 (85.71)	**42 (15.22)**	37 (21.02)	139 (78.98)	**176 (19.89)**	**0.31**	**0.18**
A. (*Ny.*) *braziliensis*	11 (23.40)	36 (76.60)	**47 (17.03)**	23 (34.85)	43 (65.15)	**66 (7.46)**	**0.19**	**0.06**
A. (*Ny.*) *darlingi*	26 (26.00)	74 (74.00)	**100 (36.23)**	46 (41.82)	64 (58.18)	**110 (12.43)**	**0.39**	**0.08**
A. (*An.*) *intermedius*	5 (23.81)	16 (76.19)	**21 (7.61)**	1 (16.67)	5 (83.33)	**6 (0.68)**	**0.01**	**0.01**
A. (*Ny.*) *konderi*	1 (50.00)	1 (50.00)	**2 (0.73)**	-	2 (100.00)	**2 (0.23)**		
A. (*An.*) *mattogrossensis*	-	1 (100.00)	**1 (0.36)**	1 (100.00)	-	**1 (0.11)**	**0.01**	**0.00**
A. (*Ny.*) *nuneztovari* s.l.	11 (25.00)	33 (75.00)	**44 (15.94)**	7 (1.39)	495 (98.61)	**502 (56.72)**	**0.06**	**0.65**
A. (*An.*) *peryassui*	2 (33.33)	4 (66.67)	**6 (2.17)**	3 (100.00)	-	**3 (0.34)**	**0.03**	**0.00**
A. (*Ny.*) *triannulatus*	3 (23.08)	10 (76.92)	**13 (4.71)**	1 (7.14)	13 (92.86)	**14 (1.58)**	**0.01**	**0.02**
Anopheles spp.	-	-	**-**	-	5 (100.00)	**5 (0.56)**	**-**	**-**
Total (%)	**65 (23.55)**	**211 (76.45)**	**276 (100.00)**	**119 (13.45)**	**766 (86.55)**	**885 (100.00)**		

I_A = Index of Anthropophily; I_Z = Index of Zoophily.

Table III – Parameters and *p* values estimated with a Generalized Linear Model with Poisson distribution explaining the absolute abundance of *Anopheles* obtained during two years of collections in the Ilha de Santana District, Amapá, Brazil. The predictor variables were: environment (intra/peridomiciliary), species activity time, and the four species collected in greater abundance. A Poisson distribution GLM was also performed explaining the absolute abundance of *Anopheles*. The predictor variables were: feeding preference (anthropophily/zoophily), species activity time, and the four species collected in greater abundance.

	Estimate	***p*-value**	
(Intercept)	3.5607	0.00753	**
Environment (intra/peridomiciliary)	7.6928	< 2e-16	***
Time	-0.9248	0.00570	**
Species[2]	0.8744	0.00878	**
	Estimate	***p*-value**	
(Intercept)	0.24924	0.125722	
Feeding preference (anthropophily/zoophily)	1.88061	< 2e-16	***
Time	0.10241	0.000865	***
Species[2]	0.51357	< 2e-16	***

Signif. Codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

[2]Diference in abundance among the four main species collected: *A. albitarsis* s.l., *A. braziliensis*, *A. darlingi* and *A. nuneztovari* s.l.

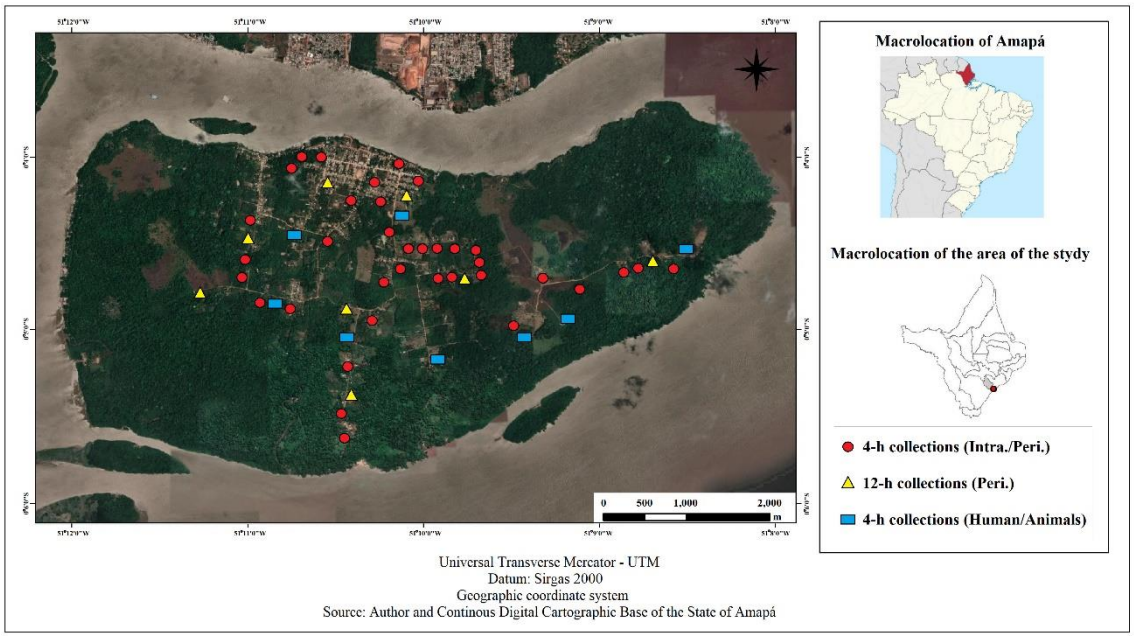

Figure 1 – Map indicating the location of the district of Ilha de Santana, state of Amapá, Brazil. In right corner is the smaller map of Brazil showing the localization of the State of Amapá (in red); Below map of the State of Amapá showing the study area (red circle). Satellite image of the district of Ilha de Santana with the 52 collection sites indicated according to the three collection categories (For interpretation of the references to color in this figure legend).

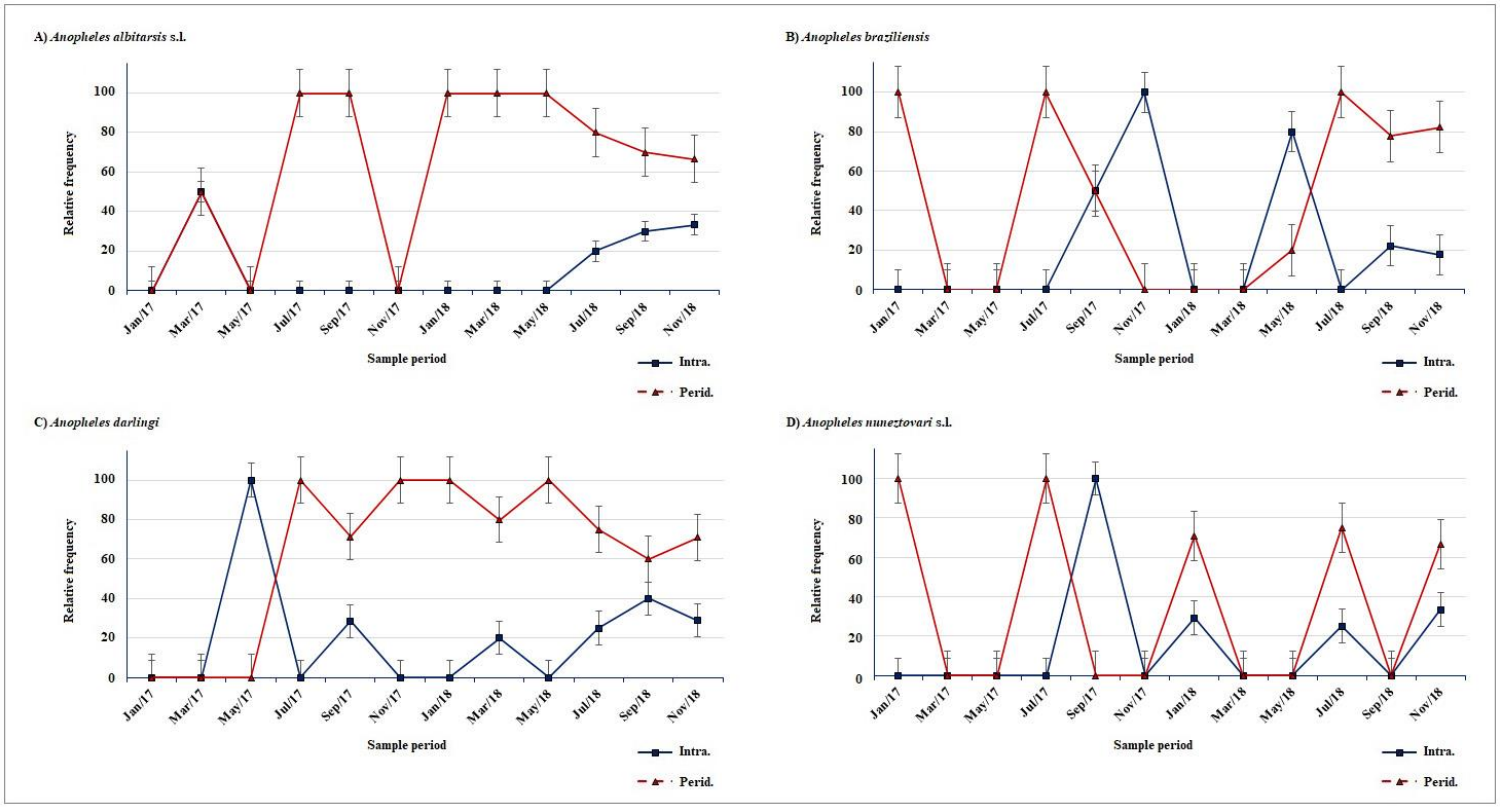

Figure 2 – Species of *Anopheles* collected indoors (intradomiciliary) and outdoors (peridomiciliary) in four-hour collections, over 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. A) *Anopheles albitarsis* s.l.; B) *Anopheles braziliensis*; C) *Anopheles darlingi*; D) *Anopheles nuneztovari* s.l. Significant difference was observed between the number of individuals collected indoors and outdoors only for *A. albitarsis* s.l. (Mann-Whitney U test = 31.5; z = -2.44; $p = 0.01$). Graph with standard deviation bars.

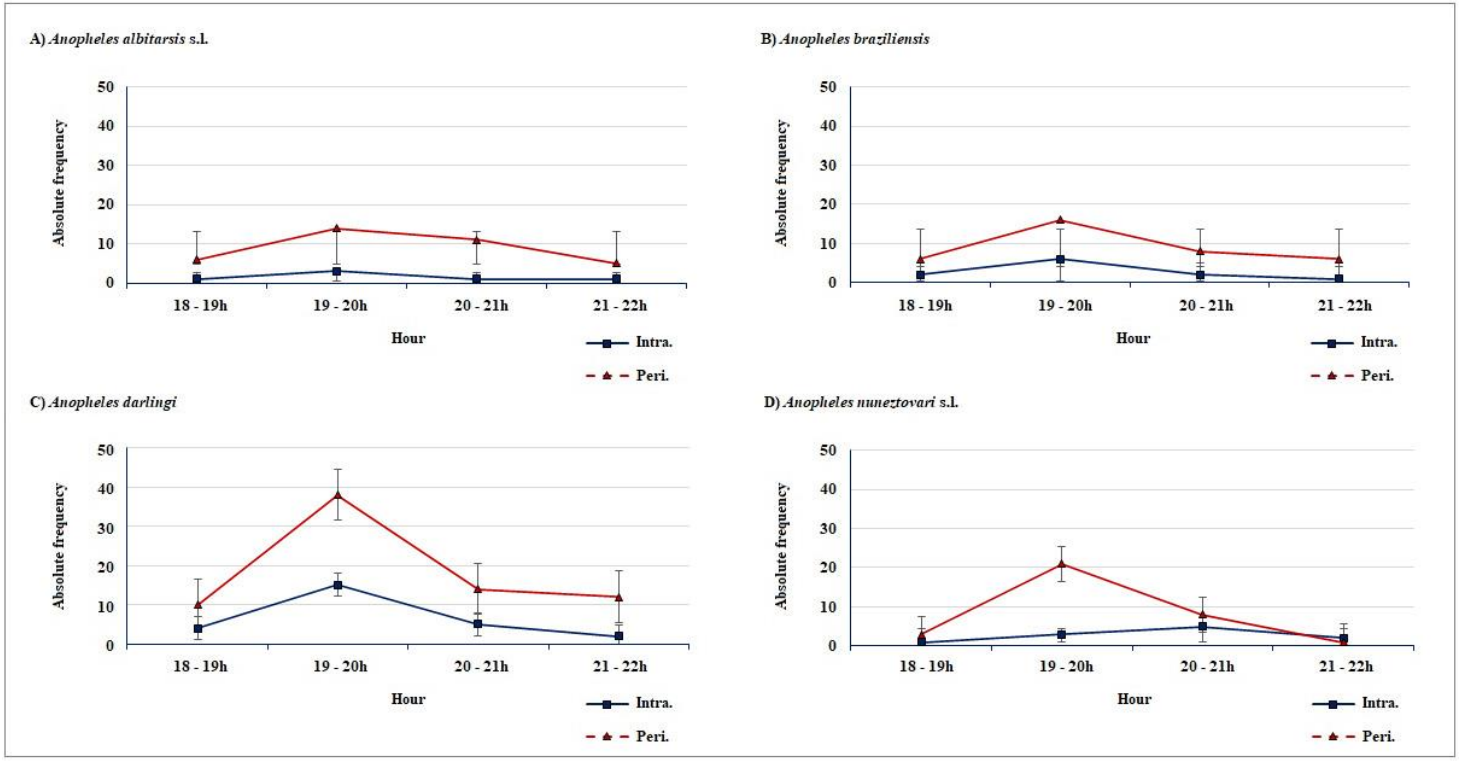

Figure 3 – Species of *Anopheles* collected indoors (intradomiciliary) and outdoors (peridomiciliary) by collection time, during 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. A) *Anopheles albitarsis* s.l.; B) *Anopheles braziliensis*; C) *Anopheles darlingi*; D) *Anopheles nuneztovari* s.l. Significant difference between indoor and outdoor environments was found for *A. albitarsis* s.l. and *A. braziliensis* (Student's *t* test [3] = -3.44; $p = 0.01$, Student's *t* test [3] = -2.49; $p = 0.04$; respectively). Graph with standard deviation bars.

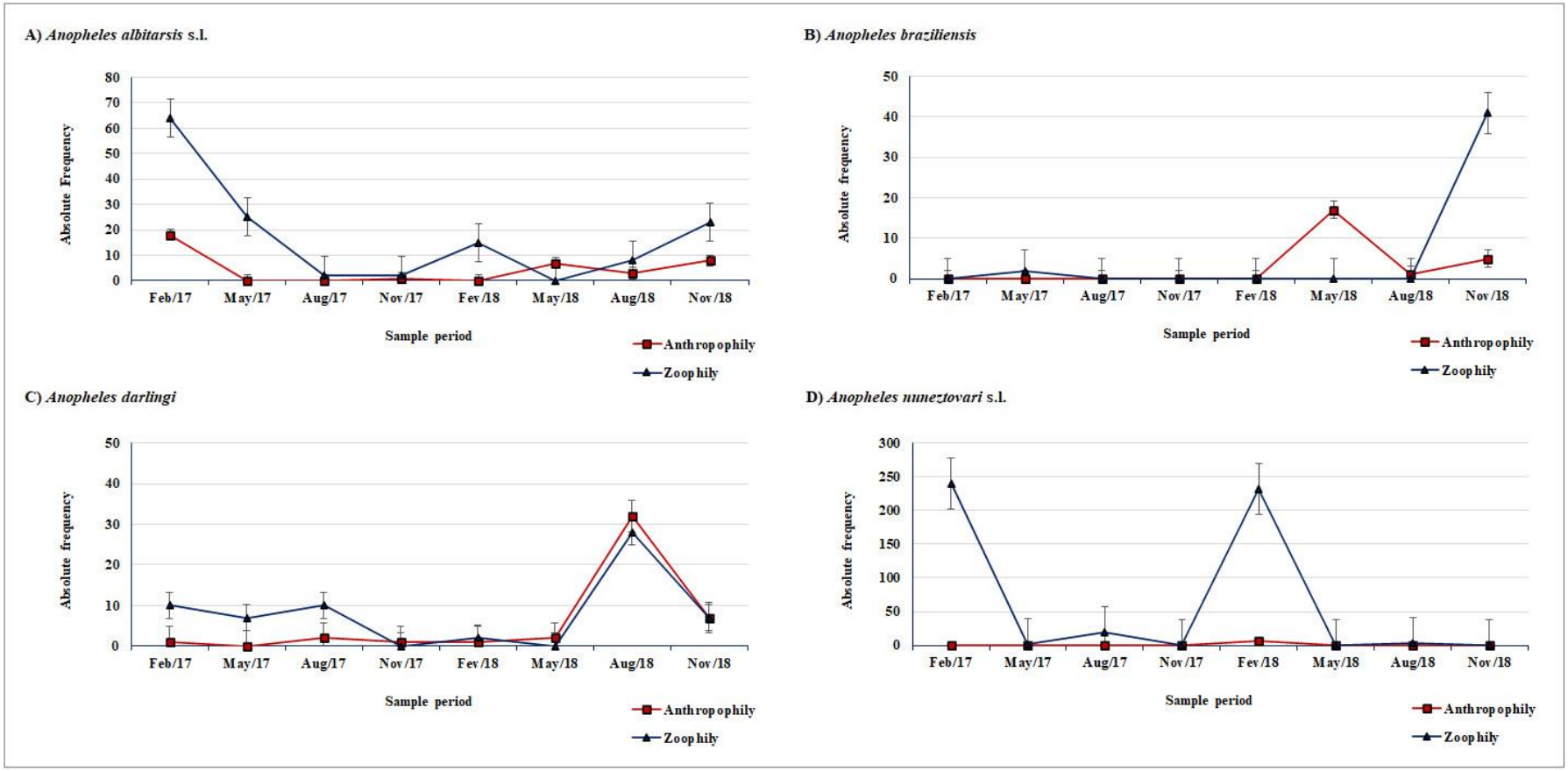

Figure 4 – Species of *Anopheles* captured in collections to evaluate anthropophilic and zoophilic behavior by month, during 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. A) *Anopheles albitarsis* s.l.; B) *Anopheles braziliensis*; C) *Anopheles darlingi*; D) *Anopheles nuneztovari* s.l. Graph with standard deviation bars.

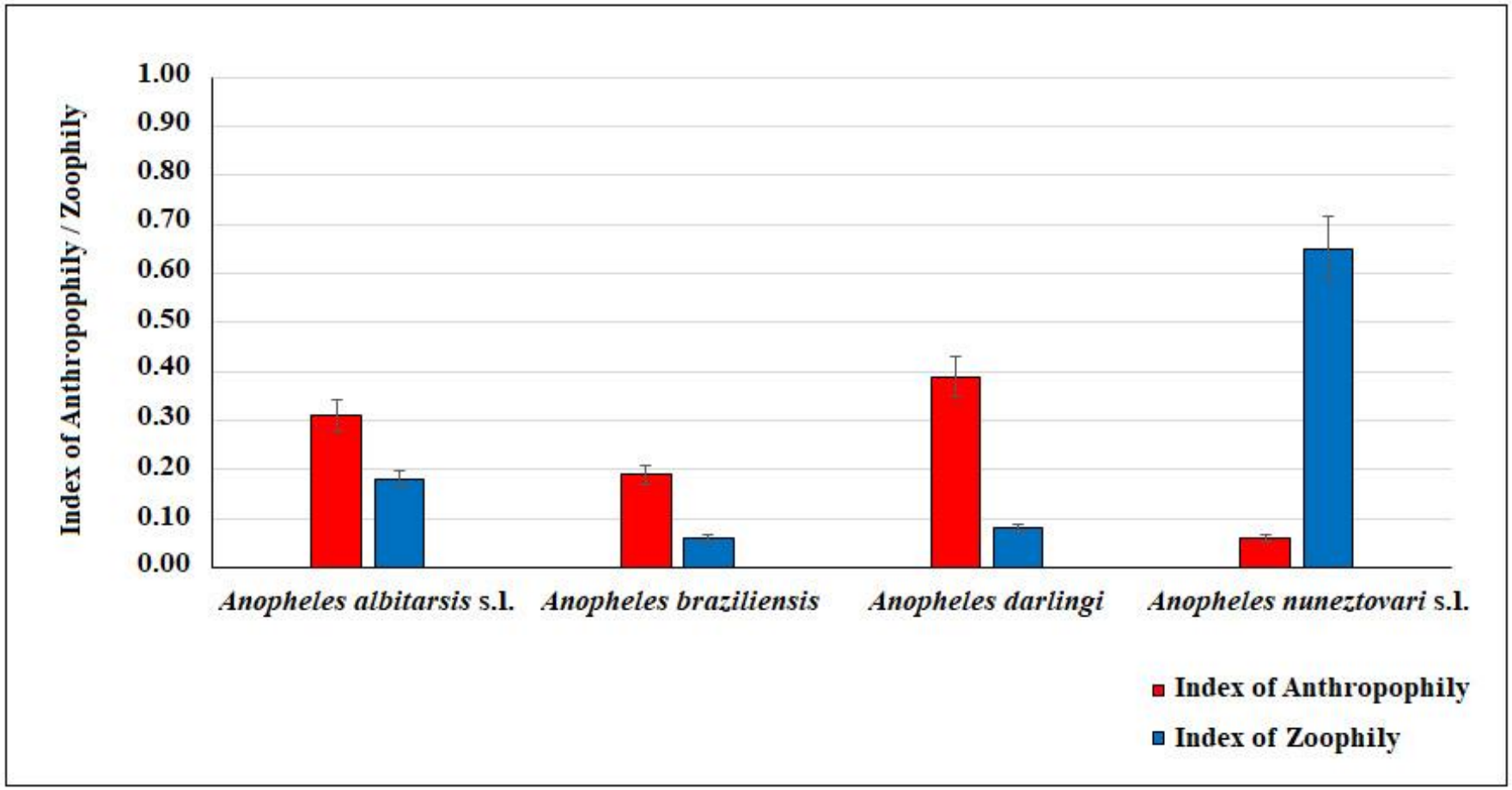

Figure 5 – Species of *Anopheles* captured in collections to evaluate anthropophilic and zoophilic behavior during 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. Graph with standard deviation bars.

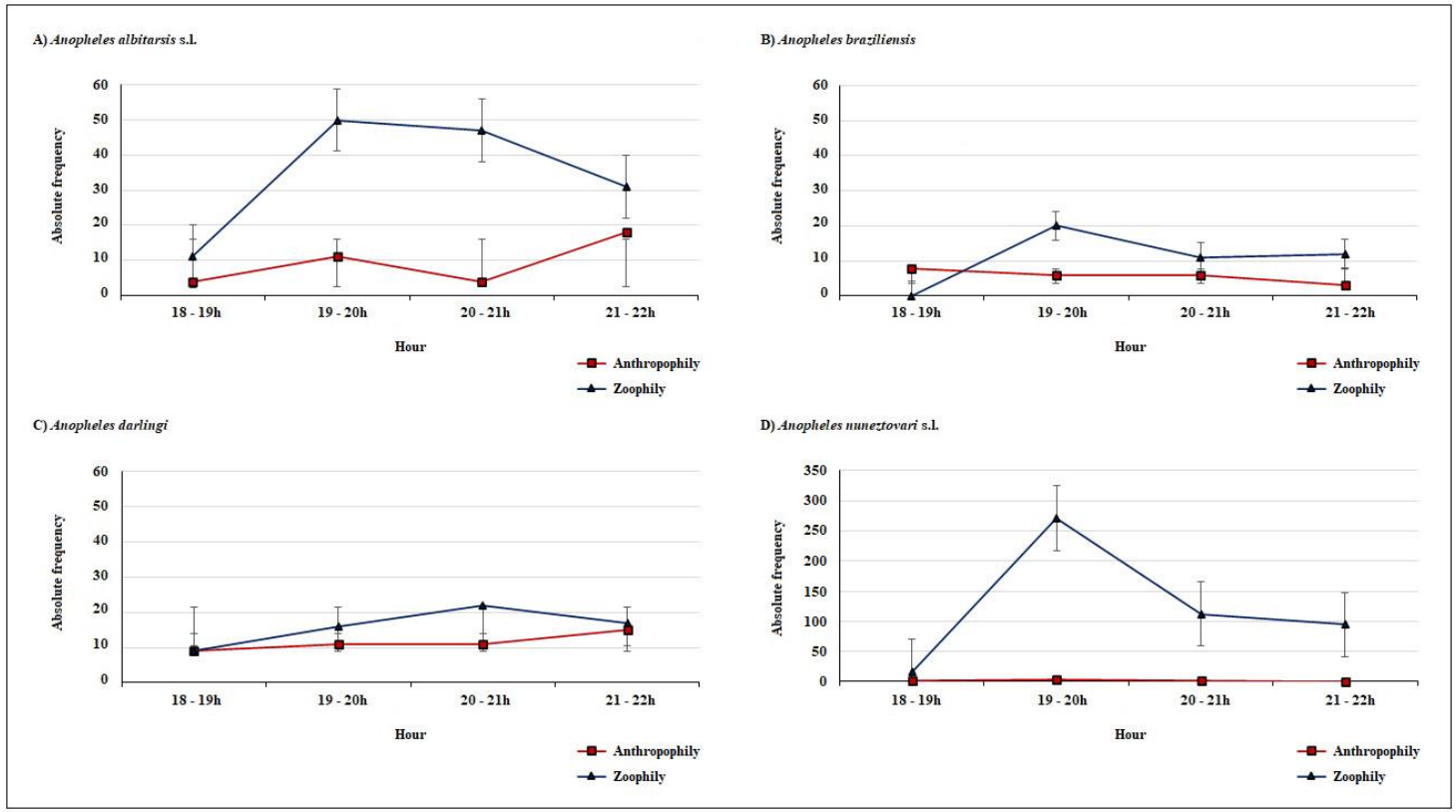

Figure 6 – Absolute frequency by time of the main species of *Anopheles* captured in collections to evaluate anthropophilic and zoophilic behavior in the district of Ilha de Santana, municipality of Santana, state of Amapá; A) *Anopheles albitarsis* s.l.; B) *Anopheles braziliensis*; C) *Anopheles darlingi*; D) *Anopheles nuneztovari* s.l. Significant difference between the number of anthropophilic and zoophilic individuals was observed only for *A. albitarsis* s.l. (Student's t test [3] = -2.67; $p = 0.03$). Graph with standard deviation bars.

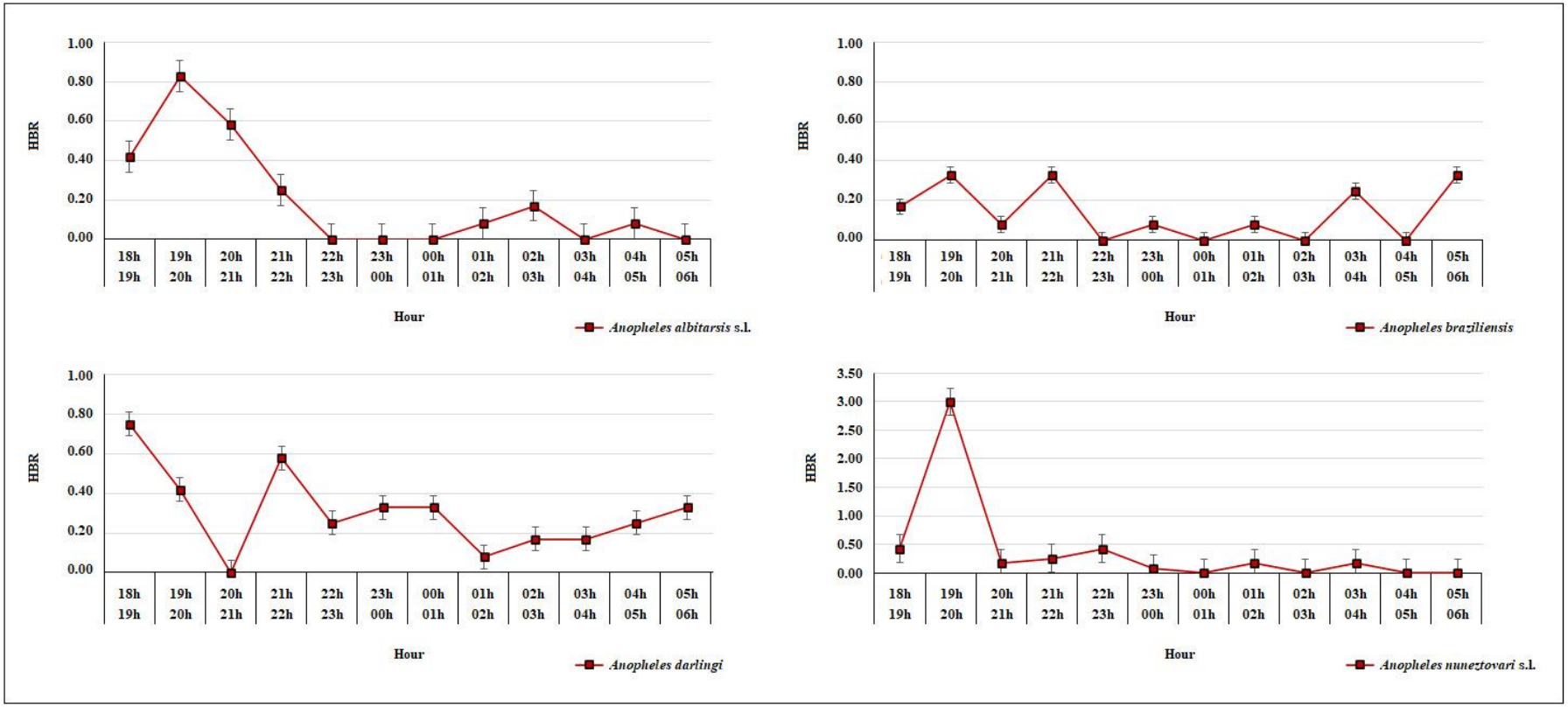

Figure 7 – Human biting rate (HBR) of the most abundant species captured in the 12-hour collections in the district of Ilha de Santana, municipality of Santana, state of Amapá. Graph with standard deviation bars.

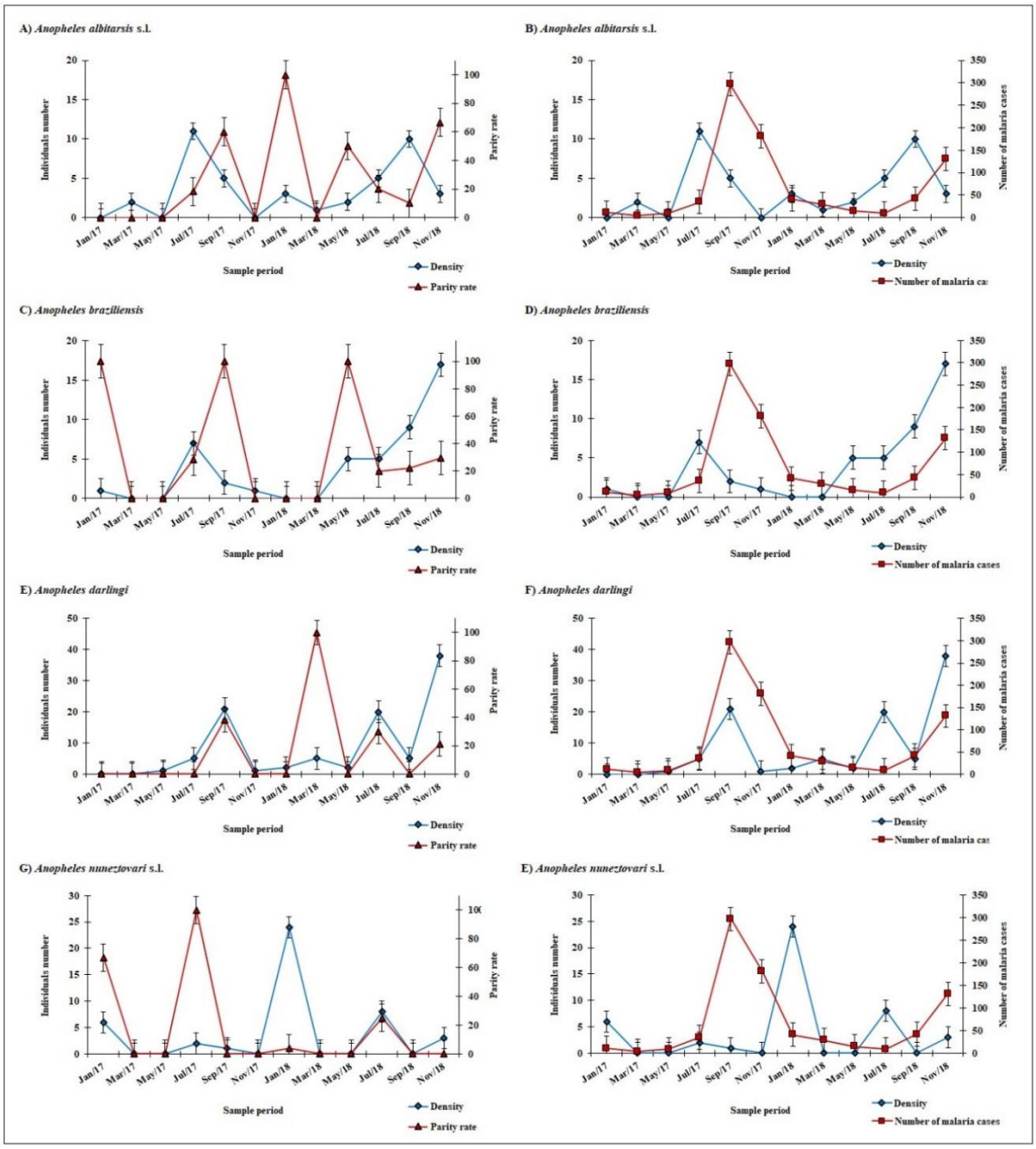

Figure 8 – Density of *Anopheles* species, parity rate, and number of malaria cases by month, during 24 months in the district of Ilha de Santana, municipality of Santana, state of Amapá. A and B) *Anopheles albitarsis* s.l.; C and D) *Anopheles braziliensis*; E and F) *Anopheles darlingi*; G and H) *Anopheles nuneztovari* s.l. The Spearman correlation between density and parity rate was significant for *A. albitarsis* s.l. (rs = 0.61; p = 0.03), *A. braziliensis* (rs = 0.80; p = 0.01), *A. darlingi* (rs = 0.72; p = 0.01) and *A. nuneztovari* s.l. (rs = 0.74; p = 0.01). Graph with standard deviation bars.

Capítulo II

Barbosa, L.M.C. & Scarpassa, V.M. 2021. Bioecology and Spatiotemporal Dynamics of *Anopheles* Meigen (Diptera: Culicidae) Species on a Malaria-endemic area of the Amazon, Amapá, Brazil. Submetido à *Revista da Sociedade Brasileira de Medicina Tropical.*

Bioecology and Spatiotemporal Dynamics of *Anopheles* Meigen (Diptera: Culicidae) Species on a Malaria-endemic area of the Amazon, Amapá, Brazil

Bionomics of *Anopheles* species

Ledayane Mayana Costa Barbosa[1],[2], *Vera Margarete Scarpassa*[2]

[1]. Universidade Federal do Amapá, Departamento de Ciências Biológicas e da Saúde, Laboratório de Arthropoda, Macapá, AP, Brasil. [2]. Instituto Nacional de Pesquisas da Amazônia, Coordenação de Biodiversidade, Laboratório de Genética de Populações e Evolução de Mosquitos Vetores de Malária e Dengue, Manaus, AM, Brasil.

ABSTRACT

Introduction: The knowledge regarding the biology and ecology of the vectors anopheline is key to identify and understand the variables that shape their distributions and consequently the transmission dynamics of malaria. The objective was to determine the faunal composition, bioecology and spatiotemporal distribution dynamics of *Anopheles* species. **Methods:** Anophelines were collected in the Ilha de Santana District, located in the state of Amapá, Brazil. To the captures of adults, the District was divided into three areas: urban, transition and rural. For collection of immatures all identified fish tanks and ponds were examined and three streams. Faunistic and ecological analyzes were carried out. In addition to simple linear regression analyses between pairs of ecological components. For the study of the spatial and temporal distributions were applied multivariate analyses. **Results:** A total of 2,278 specimens were collected, including 445

adult anophelines and 1,833 larvae. For both adults and immature phases, the same species richness and composition were obtained, distributed in nine species. In general, the most abundant species were *A. darlingi*, *A. nuneztovari* s.l. and *A. albitarsis* s.l. for collections of adults and the highest abundance was observed in the rural area. For collections of immatures the species in greater abundance again were *A. darlingi* and *A. nuneztovari* s.l. During the study the highest abundance occurred in the dry season. **Conclusions:** The risk of malaria transmission is related to temporal components, landscape fragmentation, ecological changes, anthropogenic actions, water classes (shallow and murky waters, swamps and fishponds), slash-and-burn agricultural activities, deforestation, human development index and population growth. All these factors are present in the Ilha de Santana District, making this an area of high potential for vector development and malaria transmission. Our results clearly showed that fish farming tanks are the main breeding sites for immature anophelines and are responsible for the maintenance of the malaria vector in the Ilha de Santana District.

Keywords: Fauna, Vectors Ecology. *Anopheles* larvae. Breeding Sites.

Introduction

In 2019, approximately 229 million cases of malaria were recorded worldwide, a slightly higher number than in 2018 (228 million)[1]. This disease is still one of the major public health problems worldwide[2]. Currently, ~ 480 species of *Anopheles* Meigen, 1818 are recognized and more than 50 members of species complexes not yet formally described are recognized[3]. However, worldwide, approximately 70 species of *Anopheles* can transmit malaria parasites to humans[3,4].

Anophelines occur in temperate, subtropical and tropical regions, except on most islands in the Pacific Ocean[3]. Tropical countries with poor socioeconomic conditions are more exposed to diseases, including malaria[5]. The transmission of malaria in South America is concentrated in the Amazon region, occurring in all Amazon countries[6]. In Brazil, ~ 99.8% of autochthonous cases occur in states located in the Amazon, which is considered a malaria-endemic area in the country[7]. This region has ideal environmental characteristics that favor the development and survival of anophelines, and frequent environmental changes alter the behavior of vectors and, consequently, the pattern of malaria transmission[8]. These changes in natural ecosystems alter the ecological conditions of important vectors; therefore, knowledge regarding the biology and ecology of the vectors is key to identify and understand the variables that shape the distributions of these species and consequently the transmission dynamics of this disease[9,10]. Therefore, studies on the diversity, density and spatiotemporal distribution of anopheline species are essential to describe the relationship between vectors and the incidence of malaria, as they provide support for projects aimed at controlling and mitigating the disease[11-14].

The main factors that can help elucidate the dynamics of disease transmission are the diversity and bionomy of vectors in endemic areas[15]. Immature anophelines have been reported to occur both in natural water bodies and in fish farming tanks and dams in malaria-endemic areas in the Amazon[16]. Fish farming tanks were also identified as breeding sites of *A. darlingi* Root, 1926 in the northeastern Peruvian Amazon[17]. Currently, aquaculture is a common economic activity in the Amazon region that can alter the dynamics of malaria transmission[18]. Identifying the factors that affect the population structure and the preferred larval habitat of the species, which can vary greatly, and determining the geographic distribution of vectors in endemic areas provide information that can assist in locating oviposition, which is essential knowledge for vector surveillance in entomological and epidemiological studies, providing support for the development of integrated strategies in the combat and control of vectors[16,19,20].

This study is the first in the state of Amapá to describe the relationship between the spatiotemporal distribution patterns of *Anopheles* species. The objective was to determine the faunal composition, bioecology and spatiotemporal distribution dynamics of *Anopheles* species, with an emphasis on malaria vectors in the Amazon, and their implications for vector control strategies.

Methods

Study area

Anophelines (adults and immatures) were collected in the Ilha de Santana District, located in the municipality of Santana, which comprises an area of 20.06 km^2 and is located between the 00°04’00’’ and 00°06’00’’ south latitudes and 51°08’00’’ and 51°12’30’’ west longitudes, state of Amapá, in the northernmost part of Brazil[21]. This

district is inserted in the Estuary and Delta Plain of Amapá geomorphological unit, Coastal Plain subdivision, consisting of an extensive strip of sandy, clayey and silty sediments of fluvial-marine origin. It is under fluvial influence of the Amazon River and periodic floods formed by rainwater and river floods[22]. The climate is hot and humid, with two well-defined seasons: rainy season (January to June) and dry season (July to December). The average annual temperature is approximately 27.54 °C, the average humidity is 70.81% and the average rainfall is 2,527.60 mm[23,24].

The main economic activity of the Ilha de Santana District is related to the primary sector: agriculture, livestock and extractivism. However, although there is no commercialization of fish, several fish farming tanks exist there, but none of them had fish when the present study was conducted.

The data for the climatic variables used for the analysis of the temporal distribution during the collections of adult females were measured on-site every hour: air temperature (%) and relative humidity (%), using an Incoterm digital thermo-hygrometer, model 7663. For monthly analysis of the variables mean temperature, mean relative humidity, cumulative precipitation (mm), insolation (h) and wind speed (m/s), data were provided by the Center for Hydrometeorology and Renewable Energy (NHMET) of the Institute of Scientific and Technological Research of the State of Amapá (IEPA) and collected at the Fazendinha District station, which covers the Ilha de Santana District. Epidemiological data for the number of malaria cases during the study period in the area were obtained from the Malaria Epidemiological Surveillance Information System of the Ministry of Health[7].

Sampling, processing and identification of adult females

The adult captures were performed from January 2017 to December 2018, totaling two years of observations. These captures were performed by a team of six experienced and duly trained collectors. In these captures, all team members used personal protective equipment (PPE), as established by the Ministry of Health[25]. The anophelines were collected with the aid of a flashlight and a Castro sucking tube with human landing trap, i.e., the mosquitoes were sucked in when attempting to land on the collectors[26].

Two sampling modes were adopted: (1) sampling for four hours and (2) sampling for 12 hours. Collections with a duration of four hours began at 6:00 pm and ended at 10:00 pm. These captures were performed bimonthly, six in the rainy season and six in the dry season, totaling 12 campaigns. For captures, the Ilha de Santana District was divided into three areas: urban area, transition area and rural area. The urban area is characterized by the village region, the transition area is characterized by the peripheral region (between the village and the rural area) and, finally, the rural area comprises the most distant region, characterized by rural properties. In each campaign, collections were performed at three different points, with one point selected in each area, totaling 36 collection points, 12 points per area, with two collectors at each collection point (**Figure 1A**). Collections performed for 12 hours started at 6:00 pm and ended at 6:00 am the next day, every three months, with four collections performed in the dry season and four in the rainy season, totaling eight campaigns.

The selection of the points was based on the following criteria: highest number of notifications of malaria cases; higher population density; and coverage of the largest area possible. After collection, the mosquitoes were placed in plastic cups labeled with the location, date, time and sampling mode and transported to the Arthropoda Laboratory of

the Federal University of Amapá (UNIFAP), in Macapá, state of Amapá, where they were identified. The anophelines were killed in a -20 °C freezer and identified using a Zeiss stereomicroscope, model Stemi DV4, identified using dichotomous keys[27-29]. The nomenclature adopted was that of Guimarães[30].

The captured species that composed species complexes were subjected to molecular analysis to identify their respective members, such as *A. albitarsis* s.l. Lynch-Arribálzaga, 1878, *A. nuneztovari* s.l. Gabaldón, 1940, *A. oswaldoi* s.l. (Peryassú, 1922) and *A. triannulatus* s.l. (Neiva and Pinto, 1922), using the DNA barcode region of the mitochondrial DNA *COI* gene. From each species recognized as a complex, 3% of the total captured for such analyses was sampled. Genomic DNA was extracted from the legs of mosquitoes using the phenol-chloroform method[31]. The DNA barcode region of the *COI* gene was used in these analyses (Folmer region) (data no shown).

Based on the results generated, it was possible to molecularly identify *A. konderi* Galvão and Damasceno, 1942 and *A. triannulatus*. For *A. nuneztovari* s.l., the comparison of the sequences obtained showed 100% identity with the sequences for *A. nuneztovari* and *A. goeldii* Rozeboom and Gabaldón, 1941 deposited in GenBank, which we consider to be the same species; therefore, we denominated it as *A. nuneztovari* s.l. For the specimens belonging to the Albitarsis complex, unfortunately, we did not obtain good sequences, precluding comparisons with those deposited in GenBank. Therefore, in this study, the specimens were named *A. albitarsis* s.l. Samples of *A. braziliensis* Chagas, 1907, *A. darlingi* and *A. intermedius* (Peryassu, 1908) were also sequenced and confirmed with those deposited in GenBank.

The anophelines were collected and transported in accordance with the authorization and license of the Biodiversity Information and Authorization System

(SISBIO: registration number 52442-1). This study was also approved by the Research Ethics Committee of the UNIFAP (license number 78912617.9.0000.0003).

Sampling, processing and identification of immature

For collecting immature forms, the breeding sites in the area were surveyed based on a map showing the hydrographic network of the Ilha de Santana District obtained from the State Department of the Environment (SEMA). Subsequently, a pilot field trip was conducted to identify all potential breeding sites. A total of 46 fish farming tanks, three ponds and ten streams were identified. All identified fish tanks and ponds were examined. Due to the extension of the streams, two selection criteria were applied: (1) near inhabited sites and possibility of access, given the absence of roads or trails connecting to the more distant (uninhabited) regions; and (2) preservation of streams because during the on-site survey, excess garbage dumped in some streams was observed, and they were even used for the disposal of human waste, especially those located near the urban area of the island. As mosquitoes of the genus *Anopheles* are quite demanding regarding water conditions, streams that did not meet these conditions were excluded, leaving only three (**Figure 1B**).

The collections were performed monthly from July 2019 to June 2020 at all potential breeding sites identified and always during the early hours of the morning. Immature forms were collected at the breeding sites with the aid of a standard "entomological ladle" with a 1.0-m handle, which made it possible to conduct sampling in places difficult to reach; at its end, there was a ladle-shaped aluminum frame, with diameter of 11.0 cm and a volumetric capacity of 350 milliliters[32]. The collections were systematized, following the guidelines of Technical Note no. 012 - CGPNCM/DIGES/SVS/MS, of June 4, 2007, which recommends the standardization of the methods used in *Anopheles* larval surveys[32].

A sampling point was set up every 5.0 m at breeding sites. The fish farming tanks and ponds were examined along their length, and the time used to examine the whole area was recorded. For streams, to account for the greater area and to try to adjust the sampling effort, the collection time adopted was the maximum time used during the sampling of the largest area of the other larval habitats, which was a fish farming tank (A = 100 m^2). This sampling strategy was adopted as a measure to overcome the limitations regarding the differences in size and shape between the types of breeding sites, based on a recommendation in the Manual of Malaria Entomology[19]. Only the perimeters of the water bodies were sampled as, the larvae usually remain on the margins of breeding sites[33]. For each sampling point, nine samples were taken, three to the right, three to the left and three to the front, within a radius of 1.0 m from the point set by the collector[32]. To perform the collections, all members of the team used rubber boots and protective gloves[19].

The immature forms collected were transferred to a tray and selected based on the larval stage and placed in plastic bottles labeled with the date and the number of the breeding site. Third-stage and fourth-stage larvae were preserved in 70% ethanol solution for subsequent species identification. First-stage and second-stage larvae were kept alive in plastic flasks containing water from the breeding site and fed macerated fish food (Alcon® Pet Gold Fish Colour) for development until reaching the fourth stage for identification. The pupae were also kept in flasks containing water from the breeding site to facilitate the emergence of adults for later identification. The identification of newly emerged larvae and adults was performed using dichotomous keys[27,28,34,35]. The same nomenclature and abbreviations for the generic names used for adults were applied to the larvae.

Statistical analysis

A database was generated with all the studied variables in Excel in Office 2019, providing support for the statistical analyses. To faunal analysis were estimated richness, abundance, frequency and constancy[36,37]. Richness was calculated using the nonparametric *Jackknife 1* and *Chao 2* algorithms, and a species accumulation curve was constructed to assess whether the sampling method was sufficient, with a 95% confidence interval. For analyses of the immature forms, the index of affinity was also applied for all species collected[38]. This test identifies objective groups of recurrent species, defined by the strength of their association, i.e., whether the species cooccurred more frequently than expected by chance, determined by the formula $\frac{J}{\sqrt{Na.Nb}} - \frac{1}{2\sqrt{Nb}}$, where *J* is the number of co-occurrences; *Na* is the total number of occurrences of species A alone; and *Nb* is the total number of occurrences of species B alone; the species are organized in pairs so that Na ≤ Nb. An index of affinity ≥ 0.5 indicates affinity between species.

The Shannon diversity index, Berger-Parker dominance index and Pielou evenness index were the ecological indices estimated. Simple linear regression analyses were performed between pairs of ecological components.

The multivariate analyses applied to the study of the spatial and/or temporal distributions were nonmetric multidimensional scaling (nMDS), principal coordinate analysis (PCoA), permutational analysis of variance (PERMANOVA) and similarity percentage analysis (SIMPER).

Multivariate analyses (PCoA and nMDS) were performed based on the Jaccard, Sørensen, and Bray-Curtis similarity indices and Euclidean distance. (1) The Jaccard index was selected because it uses binary qualitative coefficients (presence/absence), which is ideal for comparing communities and areas, regardless of the sample size, and

was used for the analysis of adults among the three areas (urban, transition and rural) and of the immature forms to evaluate the similarity of the populations obtained from the breeding sites, indicating the proportion of species shared among the three different types of larval habitats (fish farming tanks, ponds and streams) relative to the total number of species. Thus, the possible differences in sampling caused by different sample sizes at different breeding sites were eliminated. (2) The Sørensen index, which uses qualitative data (presence/absence) for ordination, was applied for the analysis of species composition. (3) The Bray-Curtis index was used to construct a similarity matrix based on quantitative data, i.e., relative abundance of species, used for the distribution analysis. For the analysis of larval distribution, this index was applied only when the three types of larval habitats were analyzed individually. (4) Euclidean distance, which is an appropriate metric for environmental data measurements[39], was used to analyze the temporal distribution using data for monthly abundance and the climatic variables precipitation, temperature and humidity for adults and precipitation, insolation and wind speed for immature forms. This combination of distance measures and ordination techniques is one of the best ways to describe ecological gradients based on species occurrence data[40-42].

To analyze the spatial distribution, the following were constructed: a distribution map of the area with the four species collected in greater abundance (adults and immature forms) and a kernel density map; the latter is a powerful tool for spatial analysis, constructed from the geographic coordinates of larval habitats and larval production in each habitat. A color scale was used in the generated kernel map, divided into 14 classes, grouped into high, medium and low density. The maps were prepared using ArcGIS software version 10.6.

Then, PCoA was performed with PERMANOVA to test for significant differences among the types of larval habitats. The quality of the graphical representation generated by nMDS was evaluated using the STRESS value (Standard Residual Sum of Squares). Similarity analysis (ANOSIM) was applied to test the significant differences in community structures and to assess the significance of the distribution of species that are under the influence of climatic variables. Simple and multiple linear regression analyses were performed to determine how these variables influence and can be predictors of *Anopheles* (adults and immature forms) density. These analyses were also applied to assess the influence of the same variables on the number of malaria cases as well as of mosquito density on the number of malaria cases.

The nonparametric Kruskal-Wallis test and the parametric Student's *t* test were used for comparisons of means between the ecological indices and the degrees of similarity. After the nMDS and PCoA ordinations, SIMPER was applied as a *post hoc* test to identify groups of species, areas and habitats that contributed the most to the similarities or dissimilarities.

All tests were generated in PAST–Paleontological Statistics, version 1.34[43], except for the regression analyses, which were performed in BioEstat version 5.0[44]. The descriptive statistics are presented as the mean, and the significance level used in the tests was 0.05.

Results

A total of 2,278 specimens were collected over three years, including 445 adult anophelines and 1,833 larvae. For both adults and immature forms, the same species richness and composition were obtained, distributed in nine species; of these, three

(33.33%) belonged to the subgenus *Anopheles* and six (66.67%) to the subgenus *Nyssorhynchus*. The species accumulation curve showed a tendency to stabilize, reaching an asymptote at the 30th sampling point for adults and at the 36th sampling point for immature forms. Based on the estimators *Jackknife 1* and *Chao 2*, the maximum richness was obtained with nine species, with a standard deviation of 0 and 0.046 for adults and 0 and 1.148 for immature forms (**Figure 2**).

Faunal and ecological analyses – adults

Of the total adult individuals collected, 276 (62.02%) were obtained in four-hour collections and 169 (37.98%) in 12-hour samplings. In general, the most abundant species were *A. darlingi* (32.36%), *A. nuneztovari* s.l. (22.47%) and *A. albitarsis* s.l. (15.96%). These three species were the only constants in both sampling modes (**Table 1**). There was no significant difference in species abundance between 4-hour and 12-hour collections (Student's *t* test*: p* = 0.358).

The highest abundance of adult anophelines was obtained in the rural area, with 167 (60.51%) specimens, and the lowest abundance was obtained in the urban area, with only 50 (18.11%). The abundance of *A. darlingi* and *A. albitarsis* s.l. followed the order rural area > urban area > transition area, while that of *A. nuneztovari* s.l. followed the sequence rural area > transition area > urban area. *Anopheles konderi*, *A. mattogrossensis* Lutz and Neiva, 1911 and *A. peryassui* Dyar and Knab, 1908 were restricted to a single area (rural area and transition area, respectively) and collected at low frequencies. There was no statistically significant difference in abundance between areas (Kruskal-Wallis test: $p = 0.109$).

The rural area, in addition to having the highest abundance (N = 167), also exhibited the highest richness (S = 8) and diversity (H' = 1.732). The transition area

exhibited the highest evenness (J = 0.877), and the urban area exhibited the highest dominance (D = 0.540) for the species *A. darlingi* and *A. albitarsis* s.l., which together totaled 76% of the abundance for this area (**Table 2**). Evaluating the richness per season (rainy and dry), in the four- and 12-hour collections, the highest richness was obtained in the dry season (S = 9 and S = 8, respectively). No statistically significant differences were observed among the areas (Kruskal-Wallis test: p = 0.849) or between the seasons (Kruskal-Wallis test: p = 0.106) for the ecological indices analyzed.

The highest similarity occurred between the urban and transition areas (J = 0.714), and there was no significant difference in the Jaccard similarity index among the areas (ANOSIM = 0.044; p = 0.068). By sampling mode, during the four-hour collections, the highest abundance (N = 64), richness (S = 7) and evenness (J = 1,000) were recorded in the dry season. However, the highest diversity (H'= 1.626) and dominance (D = 1.000) occurred in the rainy season (**Table 2**). A significant difference in the ecological indices tested was observed only between the months of May and November 2017 (Kruskal-Wallis test: p = 0.032). For the 12-hour collections, the highest abundance, diversity and dominance values occurred in the dry season (N = 74; H' = 1.379; D = 1.000). In general, the evenness values were high (except in August), with the highest index occurring in the rainy season (J = 1.000) and the highest richness in both seasons (S = 6) (**Table 2**). There was no significant difference among the ecological indices analyzed among the months for the 12-hour collections (Kruskal-Wallis test: p = 0.291).

When the ecological components were analyzed in pairs from the data obtained in the four-hour collections, simple linear regression revealed a strong and significant relationship between dominance and diversity [$F_{(1, 11)}$ = 17.781; p = 0.021; R^2 = 0.640] and richness and diversity [$F_{(1, 11)}$ = 30.695; p < 0.001; R^2 = 0.754], while there was a

moderate but statistically significant relationship between diversity and evenness [$F_{(1, 11)}$ = 7.776; p = 0.018; R^2 = 0.437]. Strong relationships between dominance and diversity [$F_{(1, 5)}$ = 13.036; p = 0.023; R^2 = 0.765] and between richness and diversity [$F_{(1, 5)}$ = 29.503; p = 0.006; R^2 = 0.881] were also obtained for the 12-hour collections.

Faunal and ecological analysis – immature forms

A total of 52 breeding sites were examined, of which 50 (96.15%) were positive for immature *Anopheles*. The two negative breeding sites were a fish farming tank and a stream. The species collected in greater abundance again were *A. darlingi* (N = 732; 39.93%) and *A. nuneztovari* s.l. (N = 439; 23.95%). However, unlike the adult collections, the third most abundant species was *A. triannulatus*, with 355 (19.37%), and these three species alone represented 83.25% of the total collected (**Table 1**).

Similar to the adult collections, the highest richness and abundance values were obtained in the dry season (S = 8; N = 312). In general, the diversity indices were quite high, with the highest value obtained in the rainy season (H' = 1.629). A single month showed a dominant species (November, D = 0.655), and the others showed high levels of evenness, with the highest value obtained in the rainy season (J = 0.928) (**Table 3**). There was no significant difference among months for the values obtained (Kruskal-Wallis test: p = 0.999). Simple linear regression performed for the ecological components resulted in a statistically significant model with a moderate relationship only between dominance and diversity [$F_{(1, 11)}$ = 10.116; p = 0.009; R^2 = 0.503].

The Jaccard index revealed that the most similar species were *A. darlingi*, *A. nuneztovari* s.l. and *A. triannulatus* (J = 1.000). These findings are corroborated by the index of affinity; *A. darlingi*/*A. nuneztovari* s.l. (21,793), *A. darlingi*/*A. triannulatus* (21,793) and *A. nuneztovari* s.l./*A. triannulatus* (13,801) presented the highest affinity

values. *Anopheles mattogrossensis* was the only species that showed no affinity. The statistical test confirmed the only significant difference in similarity values was between *A. mattogrossensis* and the other species (Kruskal-Wallis test: $p < 0.05$).

By analyzing individual larval habitat types (fish farming tanks, ponds and streams), the highest abundance and richness values for the three types of breeding sites were commonly obtained during the dry season. In turn, the other ecological components evaluated differed among habitat types regarding the period. Overall, the dominance values were low, and the evenness was quite high (**Table 3**). There were no significant differences in the values of the ecological indices obtained monthly for the fish farming tanks, ponds and streams (Kruskal-Wallis test: $p > 0.05$).

The analysis of the ecological components between pairs for the fish farming tanks showed a statistically significant model only between dominance and diversity [$F_{(1, 11)} = 10.902$; $p = 0.008$; $R^2 = 0.522$]. For ponds and streams, there was a significant relationship between dominance and diversity ([$F_{(1, 8)} = 8.525$; $p = 0.021$; $R^2 = 0.549$; [$F_{(1, 4)} = 16.303$; $p = 0.026$; $R^2 = 0.845$], respectively) and between richness and diversity ([$F_{(1, 8)} = 106.612$; $p < 0.001$; $R^2 = 0.938$; [$F_{(1, 4)} = 69.907$; $p = 0.003$; $R^2 = 0.959$], respectively).

Regarding the Jaccard index, when analyzing the types of breeding sites individually, the fish farming tanks and the ponds showed a high similarity for *A. darlingi*, *A. nuneztovari* s.l. and *A. triannulatus* (J = 1.000). However, these species showed no similarity in the streams (J = 0.000), revealing the greater similarity between *A. albitarsis* s.l. and *A. braziliensis* (J = 1,000) for this larval habitat. Using the Bray-Curtis index, *A. darlingi* and *A. nuneztovari* s.l. also showed similarity in fish farming tanks and ponds (BC = 0.622 and BC = 0.685, respectively). In the streams, the observed behavior was quite different, with the greatest similarity between *A. braziliensis* and *A. intermedius*

(BC = 0.800); *A. darlingi* and *A. nuneztovari* s.l. exhibited no similarity (BC = 0.000). For the Jaccard and Bray-Curtis similarity indices, there was a significant difference only between *A. mattogrossensis* and the other species (Kruskal-Wallis test: $p < 0.05$) obtained from fish farming tanks.

Spatial distribution

The main species involved in malaria transmission in the Amazon region were widely distributed in the Ilha de Santana District. Of the 27 points where adults were collected, *A. darlingi* was present at 20 (74.07%) and *A. albitarsis* s.l. at 15 (55.56%) points (**Figure 3A**). For the immature forms, of the 50 positive breeding sites sampled, *A. darlingi*, *A. nuneztovari* s.l. and *A. triannulatus* were present at 47 (94.00%).

As most of the identified breeding sites consisted of fish farming tanks and a single site had several breeding sites, for the construction of the species distribution map, the breeding sites were grouped according to their proximity. Thus, eight groups were generated. *Anopheles darlingi*, *A. nuneztovari* s.l. and *A. triannulatus* were present in all clusters (**Figure 3B**). *Anopheles albitarsis* s.l. was present in five but was collected at a low density when compared to the other three species. The highest concentration of breeding sites was observed in the transition area. In a single cluster, 19 larval habitats were found, including 18 fish farming tanks and one stream, with a total of 443 individuals collected. Two groups containing 10 breeding sites each were located in rural areas, and the three ponds studied were included in these groups (**Figure 3B**). The kernel map confirmed that the area of influence of the vector was quite wide; of the eight clusters, six were classified as high or medium risk. The breeding sites with the highest larval density and considered high risk were concentrated in the transition and rural areas (**Figures 3C and D**).

The Sørensen index, used to evaluate the similarity of species composition among the three areas of the Ilha de Santana District, showed that the distribution was similar among the areas. However, the ordination with the Bray-Curtis index revealed that the abundance of species in the urban area differed from that in the other areas, while the transition and rural areas had similar species distribution patterns. The similarity analysis was not significant for either index tested along the ecological gradient between the areas (ANOSIM = 0.045; p = 0.067; ANOSIM = 0.037; p = 0.165, respectively). Based on SIMPER analysis, the species with the highest contribution in all areas was *A. darlingi* (33.06%).

nMDS was applied to analyze the distribution of species throughout the sampling period for four- and 12-hour collections. *Anopheles darlingi*, *A. albitarsis* s.l. and *A. nuneztovari* s.l. were the species that exhibited the highest similarity. There was a significant difference in species similarity in the four-hour collections (Kruskal-Wallis test: p = 0.014) and in the 12-hour collections but, in the latter, only between *A. albitarsis* s.l. and *A. intermedius*, *A. intermedius* and *A. triannulatus*; and *A. konderi* and *A. triannulatus* (Kruskal-Wallis test: $p < 0.05$).

For immature forms, PCoA revealed a clustering of the three types of larval habitats, especially among fish farming tanks with a strong distribution pattern, in regard to species composition. Based on PERMANOVA, there was a significant difference only in the distribution of immature forms obtained from fish farming tanks and ponds (F = 4.235; p = 0.017). SIMPER analysis indicated that the species with the highest contributions were *A. darlingi* and *A. nuneztovari* s.l.; together, they accounted for 61.78% of the contribution to the breeding sites. Regarding the composition and distribution of the species throughout the sampling period, a high degree of similarity was

observed for *A. darlingi*, *A. nuneztovari* s.l. and *A. triannulatus*. Even when the types of breeding sites were analyzed individually, these three species maintained a high degree of similarity in fish farming tanks and ponds. However, these species did not show good ordination when composition and distribution were analyzed for streams. Only *A. albitarsis* s.l. and *A. braziliensis* exhibited good clustering in this habitat. In both composition and distribution analyses, *Anopheles matogrossensis* was the most dissimilar species. There was no significant difference in the composition and distribution among species throughout the sampling period or for the composition/distribution of species in fish farming ponds, ponds and streams (Kruskal-Wallis test: $p > 0.001$).

Temporal distribution

For adults, monthly clustering analysis (nMDS) was performed with the climatic variables precipitation, temperature and humidity. For both sampling modes, a relationship between the gradients and the anopheline assemblage structure was obtained. The results revealed a strong influence of precipitation on species composition. However, November 2018 was quite dissimilar. The STRESS value obtained in nMDS for the four- and 12-hour collections showed excellent accuracy for the representations of distances (STRESS: 0.054 and STRESS: 0.042, respectively). There was no statistically significant difference in species distribution between the two sampling years for either sampling mode (ANOSIM = 0.101; $p = 0.173$; ANOSIM = 0.156; $p = 0.248$, respectively). The species that contributed the most throughout the sampling period were *A. darlingi* (four-hour collections: 29.70% and 12-hour collections: 25.21%) and *A. nuneztovari* s.l. (four-hour collections: 18.07% and 12 hours: 22.91%).

Based on the climatic variables (precipitation, temperature and humidity) obtained on-site on the day of specimen collection, multiple linear regression analysis was

performed to test whether these variables influence species distribution and whether they can be used to predict abundance over time. *Anopheles nuneztovari* s.l. was the only species that presented a statistically significant model [$F_{(3, 32)} = 5.919$; $p = 0.002$; $R^2 = 0.357$]. Precipitation ($t = -2.456$; $p = 0.019$) and temperature ($t = -4.087$; $p < 0.001$) were predictors of abundance for this species. The equation that describes this relationship is $y_{\text{(abundance of } A.\ nuneztovari \text{ s.l.)}} = 1.206 - 1.701.(\text{precipitation}) - 2.889.(\text{temperature}) - 0.611.(\text{humidity})$. Simple linear regression was also performed to test the relationship of the three-monthly variables with the number of monthly malaria cases. Precipitation [$F_{(1, 11)} = 6.387$; $p = 0.028$; $R^2 = 0.389$], temperature [$F_{(1, 11)} = 6.041$; $p = 0.032$; $R^2 = 0.376$] and humidity [$F_{(1, 11)} = 9.376$; $p = 0.011$; $R^2 = 0.483$] showed statistically significant relationships with the number of malaria cases.

For the immature forms, the variables tested were precipitation, insolation and wind speed. The ordination produced by nMDS revealed a strong clustering of months in the rainy season, with a strong influence of precipitation on the immature population; however, in the dry season, there was no cohesive ordination between months. However, wind speed and insolation had a greater influence in the dry season (especially in October and December). The generated nMDS showed excellent accuracy for distance representations (STRESS: 0.027). There was a statistically significant difference between the rainy and dry seasons regarding the distribution of species under the influence of gradients (ANOSIM = 0.528; $p = 0.001$). The SIMPER analysis revealed *A. darlingi* (79.25%) as the species that contributed most throughout the period. The clustering produced by nMDS grouped the distribution of species according to the two seasons of the year in the state of Amapá, confirming the strong influence of the environmental gradients tested.

Segmenting the habitats according to type, there was a strong clustering of the months regarding the composition of the species collected in the ponds and streams under the greatest influence of precipitation. There was no cohesive clustering of the months regarding the composition of species collected in the fish farming tanks, but interference from the climatic variables during the rainy season was observed (wind speed and insolation in June; precipitation in March, April and May). The obtained STRESS value indicated an excellent graphical representation (STRESS: 0.045). There was a significant difference among the three habitat types in species distribution during the sampling period (ANOSIM = 0.351; $p < 0.05$). *Anopheles darlingi* was the species that most contributed to the community, both when analyzing the three types of breeding sites together (68.81%) and individually (> 60.15%).

Simple linear regression indicated that the climatic variables analyzed, in fact, can be predictors of larval density, and a strongly significant statistical model was obtained for abundance and precipitation [$F_{(1, 11)} = 30.991$; $p < 0.001$; $R^2 = 0.756$], insolation [$F_{(1, 11)} = 16.629$; $p = 0.002$; $R^2 = 0.625$] and wind speed [$F_{(1, 11)} = 30.439$; $p < 0.001$; $R^2 = 0.753$]. These climatic variables were also related to the number of malaria cases during the study period. Similarly, all variables were predictive, generating a very significant model for the number of malaria cases and precipitation [$F_{(1, 11)} = 27.483$; $p < 0.001$; $R^2 = 0.733$], insolation [$F_{(1, 11)} = 20.032$; $p < 0.001$; $R^2 = 0.706$] and wind speed [$F_{(1, 11)} = 24.746$; $p < 0.001$; $R^2 = 0.712$]. Relating the abundance of immature forms with the number of malaria cases, a strong and statistically significant model was also obtained [$F_{(1, 11)} = 25.242$; $p < 0.001$; $R^2 = 0.716$].

Discussion

During the study, for adults and immature forms alike, the highest abundance occurred in the dry season. However, there was an abrupt reduction in adults in the months of September and November (period of greatest drought), while for the immature forms, the abundance decreased only at the beginning of the rainy season. Rainfall modifies the physical, chemical and/or biological conditions necessary for the development of larvae at breeding sites, generating large losses for *Anopheles* populations, in addition to altering the habitats formed on the banks of rivers caused by excessive floods that can drag the immature forms to distant sites[45,46], consequently interfering with the abundance of adults.

The Ilha de Santana District is still considered a preserved site with little anthropogenic action, yet it showed low abundance. The following may be explanatory factors: it is located near the equator and, therefore, suffers from high annual solar radiation and has the Amazon River as a geographical barrier because it is an island. However, in 2019, the Ilha de Santana District showed a very high Annual Parasite Index (API), with 103.4 cases of malaria/1,000 inhabitants[7]. The main species involved in malaria transmission were present throughout the sampling period, enough to maintain high transmission levels.

Despite the low adult density, the greatest species richness and abundance belonged to the subgenus *Nyssorhynchus*, which includes the main and secondary vectors of malaria in the Brazilian Amazon, including *A. darlingi*, *A. albitarsis* s.l. and *A. nuneztovari* s.l.[47]. *Anopheles darlingi* is considered the primary vector of malaria in the Amazon biome of South America and in several regions of the Amazon River basin[48]. In addition to the three species mentioned being collected in greater abundance, they were

present whole sampling period (both seasons), both in four- and 12-hour collections. For the immature forms, the same species richness and composition were obtained, and approximately all of the sampled breeding sites were positive for *Anopheles*. The species accumulation curve and the richness estimators confirmed that the species collected were representative of the study area and that the sampling method applied was sufficient.

Anopheles darlingi was constant and was the species that most contributed to the three study areas in both seasons, and both in the four- and 12-hour collections. Similarly, in the collections of immature forms, *A. darlingi* was the species that most contributed to the environment. This species had a wide distribution, demonstrating generalist characteristics. In general, *A. nuneztovari* s.l. was the species that contributed second most to the environment and, in terms of abundance, showed dominance during adult collections (January 2018); when *A. darlingi* was dominant in the area, this species was found at low density, and the reverse was also observed. Based on these results, it is possible that there is competition between these species or an inability of these species to coexist in the adult stage.

The cluster analysis of Sørensen similarity revealed greater similarity between *A. darlingi* and *A. albitarsis* s.l. but also showed similarity between *A. nuneztovari* s.l. and *A. darlingi*. This qualitative binary method orders the groups based on presence/absence; therefore, the similarity was high because both species were equally constant throughout the sampling period. However, based on the Bray-Curtis method, *A. nuneztovari* s.l. showed a high dissimilarity from *A. darlingi*, which, in turn, showed high similarity with *A. albitarsis* s.l. These results strengthen our hypothesis regarding the inability of *A. nuneztovari* s.l. and *A. darlingi* to coexist in the adult stage and confirms the interspecific harmonic relationship between *A. albitarsis* s.l. and *A. darlingi*. In São José de Ribamar,

in the state of Maranhão, positive coexistence of *A. darlingi* and *A. albitarsis* s.l. was also detected[49]. However, for the immature forms, a harmonic relationship was evidenced between *A. darlingi* and *A. nuneztovari* s.l., as they showed a high index of similarity and affinity, in addition to being the species that most contributed to the environment. Results similar to our findings were reported in Colombia, where nine adult *Anopheles* species were collected; the most abundant were *A. nuneztovari* and *A. darlingi*, two important vectors in that country. However, the authors found that these two species shared the same niche with other *Anopheles* species[50]. *Anopheles nuneztovari* belongs to a complex of at least five cryptic species, with Colombian and Brazilian populations being distinct species[51]. *Anopheles goeldii* is one of the members of this complex and may be involved in malaria transmission in the state of Amapá[52]. Another study, also in Colombia with adult mosquitoes, reported a richness of ten species, and *A. nuneztovari* was the most predominant, followed by *A. darlingi*[53]; however, these species also did not occur in sympatry.

The rural area showed higher richness and diversity, indicating that this area has a better ecological balance for *Anopheles* species. In ecological studies, high diversity values are associated with areas with better environmental quality[54-56]. The rural area of the Ilha de Santana District has suffered less anthropogenic action and is the most preserved, characterized by the presence of several streams and fish farming tanks that are used for oviposition and by the development of immature forms of anophelines. The urban area exhibited the lowest species abundance and richness; however, this area showed dominant species (*A. darlingi* and *A. albitarsis* s.l.), revealing the vulnerability of this area to malaria transmission and the high degree of synanthropy of these species, also observed in another study[57]. The nMDS and SIMPER analysis confirm the

dissimilarity in the species composition of the urban area relative to other areas. The transition area showed the highest uniformity in the distribution of individuals between species, although the three areas showed high values of uniformity, indicating that the maximum theoretical diversity was acquired through sampling.

Despite the occurrence of dominant species in the urban area, in general, the distribution pattern of individuals was high when evenness was observed (> 0.5). The three areas evaluated in this study represented uniform communities, demonstrating that they are less susceptible to invasion and more resistant to stresses and disturbances[58]. Despite the anthropogenic action suffered in the Ilha de Santana District, such action is still incipient due to the low population density. Thus, it has a low impact on the composition and distribution of anopheline species.

An expected pattern of inverse proportionality between diversity and dominance was observed for adults and immature forms. These data are corroborated by simple linear regression analysis, confirming the existence of a strong, very significant and negative relationship between dominance and diversity and positive relationships between richness and diversity and between diversity and evenness. In a community, the individuals distribution is high when diversity and evenness are higher and dominance is lower[59], characteristics observed in the study area. For immature forms, when the ecological components were analyzed by habitat type, the fish farming tanks showed greater dominance (*A. darlingi*) in the dry season, when most malaria cases occurred. The ponds and streams showed greater evenness between species throughout the year, and when they showed dominance, it was low. The streams had the lowest abundance and no similarity between immature *A. darlingi* and *A. nuneztovari* s.l. Several factors can influence the selection of the oviposition site by female *Anopheles* that do not usually lay in waters

with strong currents, such as streams or rivers[19]. The availability of fish farming tanks in the study area favors their selection over streams, which are influenced by tides.

The main breeding sites of *Anopheles* include swamps, rice fields, puddles, drainage ditches, irrigation channels and tree holes[19]. However, anophelines have ample capacity to adapt to various niches[60]. With the deforestation and human settlement that has occurred in recent decades, new habitats have emerged, such as dams and fish farming tanks[17]. However, these mosquitoes are very demanding regarding water conditions (clean water) and the presence of organic matter[61,62]. The immature forms of some species show a preference for specific habitats[19]. *Anopheles mattogrossensis* seems to fit this type of specificity because it was found in low abundance and most (71.43%) were found in the stream habitat. This species also showed no affinity with another anopheline, and when collected as an adult, it was the only species statistically dissimilar in terms of presence/absence and abundance relative to the other species, in addition to being the only species considered accidental, based on the constancy index. In fact, *A. mattogrossensis* has very peculiar characteristics and is quite demanding regarding the quality of the breeding site for egg laying by females, requiring environments with clear water, with partial or full sun, without submerged macrophytes, associated with flood pulses of rivers and seasonal water bodies, and usually distant from human dwellings[63]. Few breeding sites in the Ilha de Santana District fit this pattern, justifying why *A. mattogrossensis* has been collected in low abundance and in the transition area, confirming the more sylvatic behavior of this species, which has a zoophilic/exophilic habit and is rarely detected feeding on humans, commonly found near or into the forest, similar to the present study[64].

In the Ilha de Santana District, fish farming tanks are the main breeding sites for immature anophelines and are responsible for vector survival. Several factors promote the formation of potential breeding sites, such as deforestation, damming of streams and fish farming activities, and these factors help maintain permanent reservoirs of *Plasmodium*[65]. Studies conducted in the state of Acre, Brazil, concluded that fishponds are the main habitats for the immature stages of *Anopheles*[16]. These fishponds can function as permanent breeding sites for these mosquitoes and may even change the pattern of malaria transmission if appropriate management techniques are not applied[18,66]. In this study, immature forms of *A. albitarsis* s.l., *A. konderi* and *A. braziliensis* showed more restrictions regarding the type of larval habitat. It is possible that some species are able to tolerate a narrow ecological range, while others may be more widely adapted[67]. These same authors also identified *A. triannulatus* as a generalist species in a study also conducted in the Brazilian Amazon[67].

Larval density can be used to estimate the abundance and dispersion of winged forms. Based on the kernel map, there are three critical regions of high vulnerability for malaria transmission in the Ilha de Santana District. These regions of greatest vector influence are concentrated in transition and rural areas. Therefore, these areas require greater attention in the application of vector control measures.

The dry season was the most favorable period for malaria transmission, as it was responsible for the highest abundance, with dominance of *A. darlingi*, proving to be the most adapted or generalist species. The statistically significant models between dominance and diversity and between richness and diversity reveal a well-defined distribution pattern for immature forms. The result of the ANOSIM confirms the significant difference in abundance between the two seasons, being strongly influenced

mainly by precipitation, based on the clustering produced by nMDS for adults and immature forms. These findings were confirmed by linear regression analysis, revealing a very strong modulating effect of these variables on the occurrence and distribution of species and consequently on the number of malaria cases. The results of the analyses indicate that throughout the year, the rainy season receives greater interference from precipitation regarding the composition of the species than do dry months, although the species are also influenced by low precipitation. The frequency of disturbances or disruptive events, including storms or natural fires, can influence the structure of a community. Disruptive events are frequent in the Amazon region; for example, in the rainiest months, there are strong storms, and during the driest months, there are "natural" or even accidental fires (controlled burning) for soil preparation before planting, as occurs in the Ilha de Santana District. We believe that the absence of November 2017 from the cluster in the nMDS occurred due to the low frequency of *Anopheles* obtained in that month. The lowest annual precipitation was observed in October and November of that year, and consequently, intense fires occurred in the Ilha de Santana District. Fires also cause damage to assemblages and populations and affect the availability of resources[68].

Some authors described a relationship between the occurrence of *Anopheles* species and climatic factors and observed a decreasing trend in the population of anophelines during the period of greatest precipitation, with the unavailability of habitats for immature forms when the rivers were at high levels[69]. It was also found that the temperature measured on the day of collection has a strong correlation with the abundance of adult mosquitoes but did not observe a significant correlation between frequency and relative humidity[70], similar to our findings. Some studies have associated the survival and development of anophelines as significantly dependent on air temperature[71,72]. *Anopheles*

species usually adapt to hot, humid regions with high rainfall, typical of tropical regions. These characteristics influence the abundance, distribution and behavior of the vector[73,74].

The ordination of *Anopheles* populations showed that the distribution patterns are largely related to climatic variations. The results of these variations may identify a very strong modulating effect on species abundance. The greatest abundance of anophelines coincided with the peak of malaria in October, the month in which the highest annual insolation and one of the lowest rainfalls occurred. In the Brazilian Amazon, the highest rates of malaria cases often occur in the dry season, starting in August, when the river levels generally stabilize, which is essential for immature forms[10,75-77]. In the third quarter of the year (commonly warmer months in which the highest annual insolation occurs in the state of Amapá), there was an increase in the density of immature forms. One of the possible effects on immature populations during this period is the reduction in development time[78]. In November (end of the dry season), the larval density began to decrease when there was an increase in wind speed; strong winds can cause disturbances in the water level. Another study described the strong effect of wind on the dynamics of the *Anopheles* population, and the authors cite that these effects cause mortality in the larval stage due to surface waves induced by the wind[79].

The risk of malaria transmission is related to temporal components, landscape fragmentation, ecological changes and anthropogenic actions[66,80]. Other major factors are water classes (shallow and murky waters, swamps and fishponds), slash-and-burn agricultural activities, deforestation, human development index and population growth[17,66,80]. All these factors are present in the Ilha de Santana District, making this an area of high potential for vector development and malaria transmission. In a study conducted in Manaus, Brazil, a relationship was also demonstrated between the density

of immature anophelines and the peak of malaria cases during the dry season, and similar to the present study, the highest abundance was observed in fish farming tanks[20].

Our results showed that fish farming tanks are the main breeding sites for immature anophelines and are responsible for the maintenance of the vector in the Ilha de Santana District because regardless of the seasonal period, they continue providing favorable conditions for the development of immature forms, including the vector[59]. Therefore, an integrated management system for these fish farming tanks is recommended, with the goal of preventing malaria outbreaks in the area. Several methods can be adopted to control *Anopheles* populations, such as the introduction of larvivorous fish, especially periodic inspection with the removal of excess floating vegetation at the edges and throughout the surface of the tanks and the appropriate use of herbicides or biological algicides and larvicides[59,62,81,82].

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Conflict of Interest

The authors declare that they have no conflict of interest.

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Table 1: Faunal indices of adult and immature *Anopheles* species collected in the Ilha de Santana District, Amapá, Brazil.

Species	Adults				Larvae	
	4-h collections		12-h collections			
	N (%)	C	N (%)	C	N (%)	C
***Anopheles* (*Ny.*) *albitarsis* s.l.**	42 (15.22)	x	29 (17.16)	x	101 (5.51)	x
Anopheles* (*Ny.*) *braziliensis	47 (17.03)	x	20 (11.83)	z	73 (3.98)	x
Anopheles* (*Ny.*) *darlingi	100 (36.23)	x	44 (26.04)	x	732 (39.93)	x
Anopheles* (An.) *intermedius	21 (7.61)	x	7 (4.14)	y	19 (1.04)	y
Anopheles* (*Ny.*) *konderi	2 (0.73)	z	1 (0.59)	z	85 (4.64)	x
Anopheles* (*An.*) *mattogrossensis	1 (0.36)	z	2 (1.18)	y	2 (0.11)	z
***Anopheles* (*Ny.*) *nuneztovari* s.l.**	44 (15.94)	x	56 (33.14)	x	439 (23.95)	x
Anopheles* (*An.*) *peryassui	6 (2.17)	y	1 (0.59)	z	27 (1.47)	y
Anopheles* (*Ny.*) *triannulatus	13 (4.71)	y	9 (5.33)	y	355 (19.37)	x
Total (%)	**276 (100.00)**		**169 (100.00)**		**1,833 (100.00)**	

N = abundance; C = constancy; x = constant species (present in more than 50% of collections); y = accessory species (present in 25 to 50% of collections); z = accidental species (present in less than 25% of collections).

Table 2: Ecological indices of adult *Anopheles* species distributed by area and sampling mode collected in the Ilha de Santana District, Amapá, Brazil.

Adults					
	N	**S**	**H'**	**D**	**J**
Area					
Urban area	50	5	1.221	0.540	0.759
Transition area	59	7	1.706	0.254	0.877
Rural area	167	8	1.732	0.377	0.833
4-h collections					
January/2017	19	3	0.809	0.632	0.737
March/2017	4	2	0.693	0.500	1.000
May/2017	1	1	0.000	1.000	0.000
July/2017	28	**7**	1.567	0.393	0.805
September/2017	36	7	1.353	0.583	0.695
November/2017	3	3	1.099	0.333	1.000
January/2018	30	4	0.703	0.800	0.507
March/2018	10	3	0.943	0.500	0.859
May/2018	13	6	1.626	0.385	0.908
July/2018	44	6	1.496	0.455	0.835
September/2018	24	3	1.059	0.417	0.964
November/2018	64	5	1.092	0.594	0.679
12-h collections					
February/2017	6	2	0.451	0.833	0.650
August/2017	33	6	1.069	0.697	0.597
February/2018	45	6	1.311	0.422	0.732
May/2018	10	2	0.693	0.500	1.000
August/2018	1	1	0.000	1.000	0.000
November/2018	74	6	1.379	0.446	0.770

N = abundance; S = richness; H' = Shannon diversity index; D = Berger-Parker dominance index; J = Pielou evenness index.

Table 3: Ecological indices of immature *Anopheles* species distributed by type of breeding site collected in the Ilha de Santana District, Amapá, Brazil.

Larvae					
	N	**S**	**H'**	**D**	**J**
July/2019	135	7	1.503	0.356	0.772
August/2019	167	7	1.363	0.431	0.700
September/2019	257	8	1.554	0.483	0.747
October/2019	312	8	1.625	0.401	0.781
November/2019	304	8	1.135	0.655	0.546
December/2019	214	5	1.426	0.383	0.886
January/2020	117	6	1.629	0.368	0.909
February/2020	111	6	1.357	0.396	0.758
March/2020	75	6	1.554	0.373	0.867
April/2020	49	5	1.494	0.306	0.928
May/2020	23	4	1.155	0.478	0.833
June/2020	69	5	1.190	0.507	0.739
Fish farming tanks					
July/2019	117	7	1.462	0.368	0.751
August/2019	135	6	1.337	0.430	0.746
September/2019	207	8	1.561	0.478	0.751
October/2019	289	8	1.608	0.398	0.773
November/2019	275	7	1.087	0.676	0.559
December/2019	190	5	1.420	0.363	0.882
January/2020	110	6	1.630	0.364	0.909
February/2020	104	6	1.383	0.385	0.772
March/2020	71	5	1.444	0.394	0.897
April/2020	49	5	1.494	0.306	0.928
May/2020	23	4	1.155	0.478	0.833
June/2020	62	5	1.209	0.500	0.751
Ponds					
July/2019	18	5	1534	0.278	0.953
August/2019	23	3	1022	0.522	0.930
September/2019	30	5	1313	0.533	0.816
October/2019	17	5	1461	0.412	0.908
November/2019	24	3	0.990	0.542	0.901
December/2019	24	4	1146	0.542	0.826
January/2020	7	3	1004	0.429	0.914
February/2020	7	2	0.683	0.571	0.985
June/2020	7	3	0.956	0.571	0.870

Streams					
August/2019	9	4	1.273	0.444	0.918
September/2019	20	5	1.426	0.450	0.886
October/2019	6	3	1.011	0.500	0.921
November/2019	5	2	0.673	0.600	0.971
March/2020	4	2	0.562	0.750	0.811

N = abundance; S = richness; H' = Shannon diversity index; D = Berger-Parker dominance index; J = Pielou evenness index.

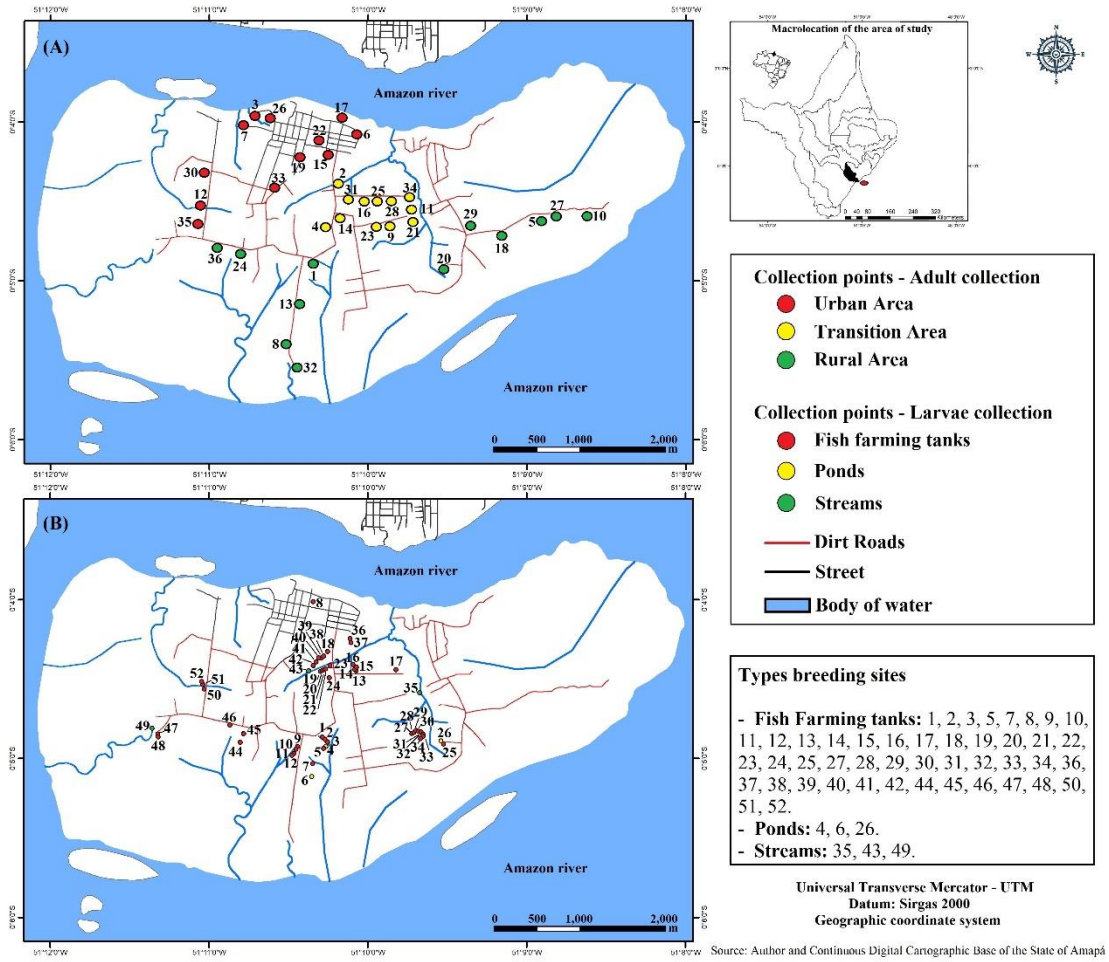

Figure 1: Map indicating the location of the district of Ilha de Santana, state of Amapá, Brazil. In right corner is the smaller map of Brazil showing the localization of the State of Amapá (in black); and map of the State of Amapá showing the study area (in red); (A) Map of the district of Ilha de Santana with the 36 collection sites (collections of adults) indicated according to the three areas: urban, transition and rural (For interpretation of the references to color in this figure legend); (B) Map of the district of Ilha de Santana with the 52 collection sites (collections of immatures) indicated according to the three types breeding sites: fish farming tanks, ponds and streams (For interpretation of the references to color in this figure legend).

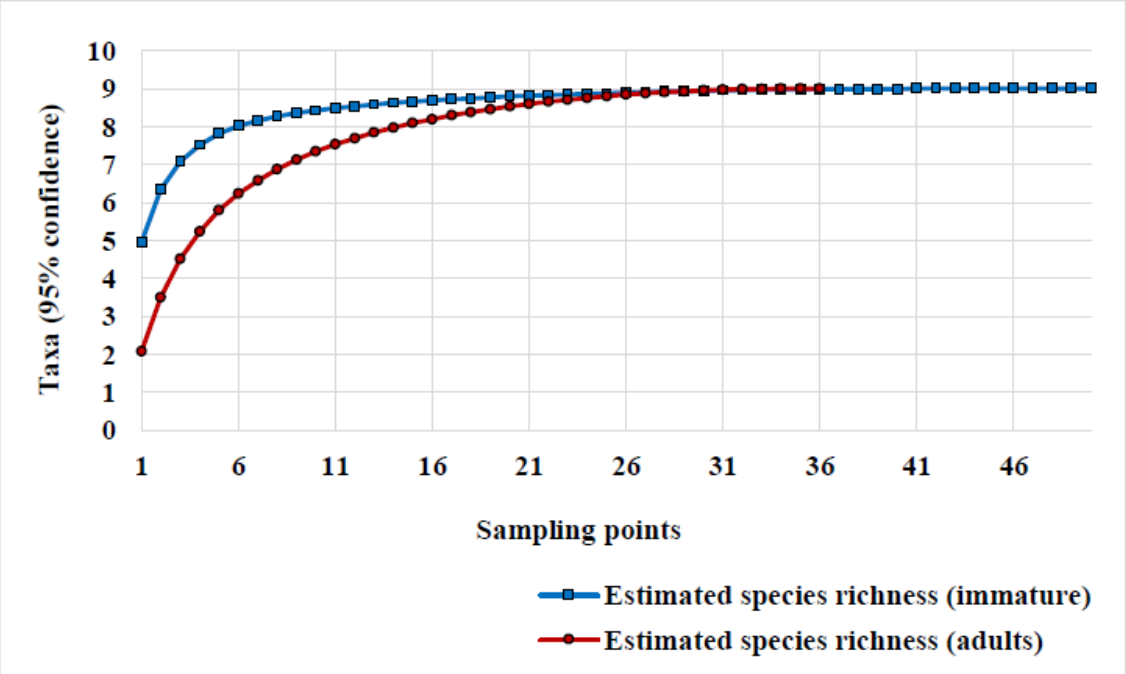

Figure 2: Species accumulation curve. The red line estimates the richness of *Anopheles* species (adults) at the 36 sampling points. The blue line estimates the richness of *Anopheles* species (immature form) at the 50 sampling points.

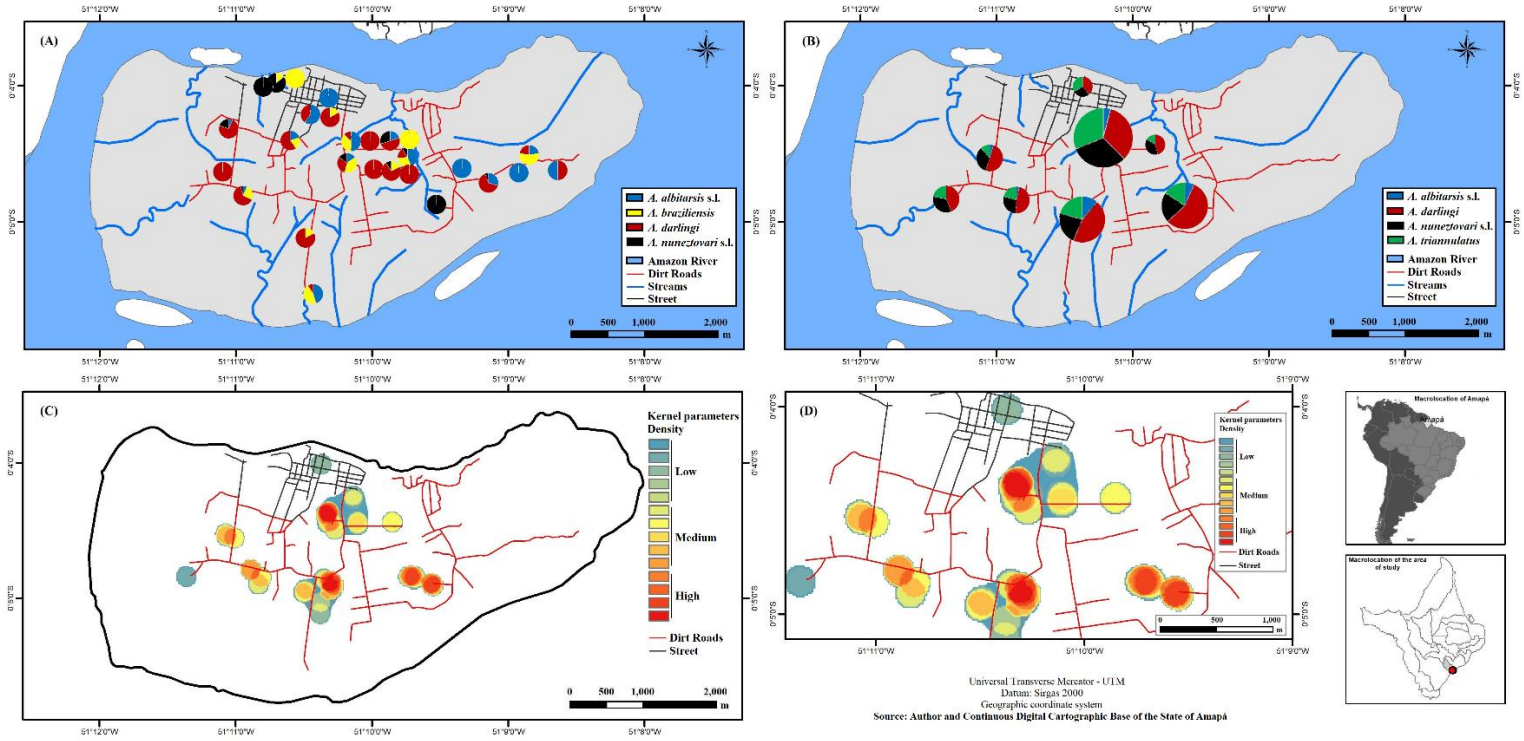

Figure 3: Hydrographic map of the Ilha de Santana District; (A) Distribution map of the four species obtained in greater abundance during the collections of adults; (B) Distribution map of the four species obtained in greater abundance during the collections of immature forms. The size of the pie chart is related to the number of breeding sites and the larval density of each cluster generated; (C) Kernel map, area of influence of the vector, according to the density of immature forms, with the nine *Anopheles* species collected by breeding sites; (D) Magnified image of the zones of influence.

Capítulo III

Barbosa, L.M.C. & Scarpassa, V.M. 2021. Bionomics of *Anopheles* species (Diptera: Culicidae) in an area of malaria transmission in the northern Brazilian Amazon. Submetido à *Acta Amazonica.*

Bionomics of *Anopheles* species (Diptera: Culicidae) in an area of malaria transmission in the northern Brazilian Amazon

Ledayane Mayana Costa BARBOSA*[ab]; Vera Margarete SCARPASSA[b]

[a]Laboratório de Arthropoda, Departamento de Ciências Biológicas e da Saúde, Universidade Federal do Amapá. Rodovia Juscelino Kubitschek, km 02 - Jardim Marco Zero, CEP: 68903-419, Macapá, Amapá, Brasil. e-mail: barbosalmc@unifap.br

[b]Laboratório de Genética de Populações e Evolução de Mosquitos Vetores de Malária e Dengue, Coordenação de Biodiversidade, Instituto Nacional de Pesquisas da Amazônia. Av. André Araújo - Aleixo, CEP: 69.067-375, Manaus, Amazonas, Brasil. e-mail: vera@inpa.gov.br

Abstract

The distribution and abundance of anopheline species are directly associated to the characteristics of breeding sites. This study aimed at identifying and structurally characterizing the preferred breeding sites of *Anopheles* species, as well as the limnological and entomological parameters of species involved in malaria transmission in an endemic area. Samples of immature stages and water of breeding sites were collected in the district of Ilha de Santana, state of Amapá, Brazil. Collections were carried out monthly from July 2019 to June 2020. From a total of 52 breeding sites identified in the study area, 20 were selected for the analysis of physicochemical parameters. Generalized Linear Models, Multivariate analyses and the Kruskal-Wallis test was applied. Of the 52 breeding sites identified and sampled, 50 (96.15%) were

positive for *Anopheles* species. *Anopheles darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* were found in 47 (94%) breeding sites. The fish farming tanks had the highest abundance (1,632 specimens). The variables type of breeding site, shading and especially type of water revealed a significant effect on *A. darlingi*. *Anopheles darlingi* was the species most affected by pH, total dissolved solids, electrical conductivity, and nitrate. A high positivity rate was found for *A. darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* (0.904). In general, our findings indicate that larval abundance was not affected by vegetation, dissolved oxygen, color, phosphate, total dissolved solids, electrical conductivity and nitrite. Of all the parameters analyzed, shading was one of the main determinants for *A. darlingi*. In conclusion, our findings suggest an important role of fishponds in the maintenance in high density vector populations throughout year in the studied area. Hence, we suggested that the implementation of targeted control measures and planning of management practices for fish farming tanks.

Keywords: Immatures; Environmental parameters; Limnological parameters; Entomological parameters.

Introduction

In Brazil ~ 99,5% of malaria cases are reported in Amazonian region, where the *Anopheles darlingi* is recognized as the main vector. However, the distribution of this disease in the region exhibits a heterogeneous pattern that is associated to diverse eco-epidemiologic profiles, such as mining, rural settlements, indigenous populations, deforestation, road and highway opens (Chaves *et al.* 2021; Rufalco-Moutinho *et al.* 2021). Thus, to study the larvae biology and ecological aspects and behavior of the adults in each one of these scenarios provide subsidies for vector control.

Female *Anopheles* mosquitoes require accumulated water to lay their eggs and allow the development of immatures. These breeding habitats are natural or artificial water bodies, such as riverbanks, lagoons, streams, marshes, with preferably clean water with organic matter, aquatic vegetation, and shading (Rejmánková *et al.* 1999; Walshe *et al*. 2017). The distribution and abundance of anopheline species, including malaria vectors, are directly associated to the characteristics of breeding sites (Mckeon *et al.* 2013).

In recent decades, the processes of occupation and anthropization in the Amazonian region have created new larval habitats, such as dams, fish farming tanks and ponds, among others (Vittor *et al.* 2009). Interest in aquaculture made stable water habitats available with ideal conditions for anopheline oviposition, in particular *A. darlingi.* Also, fish farming tanks are usually located near inhabited dwellings and provide opportunities for hematophagous mosquitoes to feed on humans (Barros and Honório 2015).

The physical and chemical characteristics of water in breeding sites has a direct influence in the metabolism and development of immatures of anopheline mosquitoes, as they promote the proliferation of algae and other micro-organisms that are essential in the diet of these immatures (Forattini 1962; Bergo *et al.* 1990; Arcos *et al*. 2018), which have interference in the larval distribution and development, as well as population adult density (Mckeon *et al.* 2013; Soleimani-Ahmadi and Vatandoost 2014). This knowledge is essential for the control of anopheline populations, especially malaria vectors.

In 2020, in Brazil 131,980 cases of malaria were reported; of these 130,455 occurred in the Amazon region (SIVEP/malaria 2021). In recent years, high annual parasite indices (API) have been observed in the district of Ilha de Santana, state of

Amapá, Brazil. In 2019, the API was 103.4 cases/1,000 inhabitants (API ≥ 50), all positive for *Plasmodium vivax* (SIVEP/malaria 2020). In addition to the high API in the region, another major factor is the absence of studies on the bionomics of anopheline immatures in the state of Amapá. Determining the factors and characteristics of larval habitats can assist the identification and location of oviposition sites and support the development of integrated strategies in the prevention and control of vectors (Reis *et al*. 2015; Arcos *et al*. 2018). This study aimed at identifying and structurally characterizing the preferred breeding sites of *Anopheles* species, as well as the limnological and entomological parameters of species involved in malaria transmission in an endemic area of the northern Brazilian Amazon.

Material and Methods

Samples of immature stages and water of breeding sites were collected in the district of Ilha de Santana (00º04'00"S and 00º06'00"S, 51º08'00'W' and 51º12'30"W), located in the municipality of Santana, state of Amapá, Brazil (Valente *et al.* 1998). Santana is situated in southern Amapá, 26 km from the capital Macapá and encompasses an area of 20.06 km^2 (IBGE 2020). The population of this district is distributed among urban, transition, and rural areas, and the main economic activities include agriculture, livestock, and extractivism. Fish farming tanks are also in found, concentrated in transition and rural areas, but currently these tanks are apparently inactive.

The vegetation of the district of Ilha de Santana is comprised of five types. **Equatorial Subperennifoliar Forest**: occurs on dry land with lush vegetation; however, the natural fertility of the soil is low. After slash and burn practices for planting, nutrients are leached, because of the disruption of the balance in the ecosystem (Brazil 1974). The

most frequent forest species are locally known as angelim-pedra (*Dinizia excelsa*), matá-matá (*Eschweilera* sp.), louro vermelho (*Ocotea rubra* Mez), itaúba (*Sílva duckei* A Sampaio), aquariquara (*Minquartia guianensis* Aubl), maçaranduba (*Manikara huberi* Ducke), and termiúba (*Goupiabra gla* Aubl.) (Valente *et al.* 1998). **Equatorial Várzea Forest:** located on the banks of watercourses, in areas influenced by fluctuations in river water level, during cycles of high and low water. It is characterized by the presence of forest species adapted to conditions of excess water, such as açaí (*Euterpe oleracea*) (Valente *et al.* 1998). The species that compose the vegetation differ from those found on dry land, such as patauá (*Oenocarpus patauá*), buriti (*Maurítia flexuosa*), murumuru (*Astrocaryum murumuru*), marajá (*Bactris* sp.), and açaí (*Euterpe oleracea*) (Valente *et al.* 1998). **Mangrove:** plant formation with high regeneration capacity in the district of Ilha de Santana composed of siriba or siriúba (*Aviceuuia* sp.), which commonly occurs in habitats influenced by tides, even in those with low salinity levels (Brazil 1974). **Equatorial Campo Cerrado:** consists of medium-sized vegetation, between four and seven meters high, of sclerophyllous shrubby species, scattered on a grassland (*Aristida* sp.) (Valente *et al.* 1998). **Juncus:** occurs in lakes, also known as Floodplain (Valente *et al.* 1998).

Environmental parameters (structural characterization of larval habitats)

Larval habitats were first identified in a hydrographic map of the study area obtained from the Secretariat of Environment of the State of Amapá (Secretaria do Meio Ambiente do Estado do Amapá - SEMA) and later confirmed in the field. The potential breeding sites were numbered, photographed, georeferenced with a Garmin Etrex Legend H GPS, and structurally characterized according to the Bulletin of Immature Collection that provides a detailed classification of larval habitats (Williams and Pinto 2012) (Figure

1). The size of the breeding site and the distance from human dwellings were measured. Subsequently, sites were categorized based on the classification of larval habitats (pond, swamp, dam, stream, ditch, swamp, dam, canal, stream, fish farming tank, fish pond, among others), shading (none, < 50% or > 50%), water type (clear, cloudy, brackish, or polluted), presence of debris (trunks/roots, leaves, fruits, and/or flowers), current (strong, moderate, weak, or null), vegetation (emergent, floating, and/or submerged) and type of the breeding site (permanent, semi-permanent or temporary). The type of water was evaluated based on color; this factor was not associated with the presence of garbage in the breeding sites, as waste was not observed in the sites evaluated.

Limnological parameters

From a total of 52 breeding sites identified in the study area, 20 were selected for the analysis of physicochemical parameters. Four water samples were collected from each breeding site, two in the dry season (November and December 2019) and two in the rainy season (February and June 2020). Water samples of the breeding sites were collected with the aid of a long dipper (described below) and transferred to sterile 500-mL autoclavable polypropylene bottles. Samples were then stored in a thermal box refrigerated at 4 °C to 8 °C and transported to the Laboratory of Water Quality Control of the Water and Sewage Company of Amapá (Companhia de Água e Esgoto do Amapá - CAESA) for analysis on the same day of sampling.

The physicochemical parameters of water samples were potential of hydrogen (pH), water temperature (ºC), dissolved oxygen (DO - mg/L), color, electric conductivity, total dissolved solids (TDS), nitrogen forms (nitrate, nitrite, and ammonia), total phosphate, and turbidity (UNT). Water temperature (°C) and dissolved oxygen - OD (mg/L) of water samples were measured in the field with an *oximeter* (Digimed, model

DM-4P). The remaining analyses were carried out in the laboratory. Potential of hydrogen (pH) was measured using the electrometric method with a benchtop pHmeter (Hach - Hexis científica S/A, model sensION^{tm} PH31). Turbidity analyses (UNT) were performed with the nephrometric method using a turbidimeter (PoliControl, model AP2000). The color of water was analyzed (method 8025) according to the APHA Platinum - Cobalt Standard Method. Analysis of total phosphate (method 8048), including nitrate (method 8039), nitrite (method 8507), and ammonia (method 8038) was performed with a spectrophotometer (Hach, model DR 3900). Finally, analysis of electrical conductivity and content of total dissolved solids (STD) were carried out with an electrical conductivity meter (Hanna, model HI 8730). All analyses of physicochemical parameters were performed according to the Standard Methods for the Examination of Water and Wastewater (APHA 2011) and the CETESB Collection and Sampling Guide (2012).

To quantify the environmental carrying capacity of a water body, the main parameters of water quality need to be evaluated with physicochemical measurements (Cunha *et al.* 2003). Water quality parameters were established following the resolutions of the National Council for the Environment-CONAMA (Resolution #357/2005 of March 17th, and Resolution #430/2011 of May 13th) which classify water bodies and provide environmental guidelines for their classification, in addition to establishing the conditions and patterns of effluent discharge. The analysis of parameters is described in Art. 42 for the classification of class 2 fresh waters (Brazil 2005; 2011).

Entomological parameters

For the analysis of entomological parameters, immature stages were collected in all breeding sites mapped in the district of Ilha de Santana (permanent or temporary and natural or artificial). Forty-five fish farming tanks, three ponds, and 10 streams were

identified. All fish farming tanks and ponds were inspected. For streams, two selection criteria were used due to their extension: (1) Near human dwellings and accessibility, due to the absence of roads or trails to the most remote regions (uninhabited); (2) Preservation of streams, as waste disposal, including human waste, was observed during *in loco* survey in some streams, especially those located near the urban area of the Ilha de Santana. As mosquitoes of the genus *Anopheles* have specific water requirements, streams that did not meet these conditions were excluded, with only three remaining.

Monthly collections were carried out from July 2019 to June 2020, totaling twelve sampling events. The collection of immature stages in breeding sites was performed in the early morning hours, every 5 m from a defined collection point throughout the entire length of the breeding site. At each point, water was sampled nine times, three on the right, three on the left, and three in front, maintaining a radius of 1.0 m from the point determined by the collector, according to the guidelines of Technical Note #012 - CGPNCM/DIGES/SVS/MS, of 4 June 2007, which standardize the methods used for studies on *Anopheles* larvae (Brazil, 2007). Immature specimens were collected with the aid of a standard entomological dipper (11 cm in diameter and 350 mL of volume capacity) with a handle of 1.0 m in length, to allow sampling in sites of difficult access (Brazil 2007). Each time water was sampled, the water volume was recorded and the contents were transferred to a tray. Third and fourth instars larvae were transferred to plastic vials filled with 70% alcohol and later identified. First and second instars larvae were placed in plastic jars with water from the breeding site and monitored until they reached the 4^{th} instar larvae to be identified; pupae were kept in plastic vials with water from the breeding site until the emergence of adults and later identified. All vials were labeled with date and breeding site number. For species identification, dichotomous keys

of Gorham *et al*. (1967) (modified), Faran (1980), Faran and Linthicum (1981) and Consoli and Lourenço-de-Oliveira (1994) were used. Because morphological keys are not able to distinguish within species complexes, molecular identification was carried out for adult specimens, such as: *A. albitarsis* s.l., *A. nuneztovari* s.l., *A. oswaldoi* s.l., and *A. triannulatus* s.l. (data not shown). The DNA barcode of the mitochondrial *COI* gene was used. Genomic DNA was extracted from the legs of mosquitoes using the phenol-chloroform method described by Sambrook and Russell (2001). Based on these results, *A. konderi* and *A. triannulatus* were molecularly identified, while for *A. nuneztovari* s.l., the sequences obtained showed 100% identity when compared to those of *A. nuneztovari* and *A. goeldii* deposited in GenBank and were then considered to be the same species and in this study, identified as *A. nuneztovari* s.l. For specimens of the Albitarsis complex, the sequences obtained could not be compared with those deposited in GenBank, and individuals were identified as *A. albitarsis* s.l. Samples of *A. braziliensis*, *A. darlingi*, and *A. intermedius* were sequenced and confirmed in the study area based on comparisons.

For mapped breeding sites located in private properties, the owner's permission was requested to collect entomological and limnological samples. The breeding sites were identified by numbers to maintain the confidentiality of owners. This study was approved by the ethics committee of the Federal University of Amapá (UNIFAP: license number 78912617.9.0000.0003), and the collection and transport of anopheline was authorized and permits were obtained from the Information and Authorization System on Biodiversity (SISBIO: registration number 52442-1).

The entomological parameters evaluated for the descriptive analysis of immatures were:

Larval Index per Man/Hour (LIMH) to estimate larval density expressed by the equation: $LIMH = \sum_{j=1}^{L} \frac{N}{\frac{(Cxh)}{S}}$

Where: *N* = number of larvae; *C* = number of collectors; *h* = number of hours of collection; and *S* = number of collection sites (Tadei *et al.* 2007).

Positivity Index of immature forms (PI) estimated by the formula: $PI = \frac{PBs}{TB}$

Where: *PBs* = number of positive breeding sites for a given species; *TB* = total number of breeding sites surveyed.

General Breeding Index (GBI), which is a measure of the ratio of water collections that mosquitoes were found developing in a given locality, determined by the equation: $GBI = \frac{PB}{TW}$

Where: *PB* = number of breeding sites with anopheline immatures (positive breeding sites); *TW* = total number of water collections obtained.

Absolute Breeding Index (ABI), which is the relative ratio of breeding sites occupied by a vector species in a given locality, expressed as: $ABI = \frac{PBs}{NW}$

Where: *PBs* = number of positive breeding sites for a given species; *NW* = total number of water collections obtained.

Relative Breeding Index (RBI), which reflects the abundance of breeding sites of a given species compared to the number of water collections where mosquitoes were found in a locality, estimated by the formula: $RBI = \frac{PBs}{TPB}$

Where: *PBs* = number of breeding sites positive for a given species; *TPB* = total number of positive breeding sites.

Statistical analysis

Generalized Linear Models (GLM) were applied according to the characteristics and distribution of the dependent variable. The most appropriate GLM for the analysis of environmental parameters was the multinomial logistic regression which evaluates the relationship of categorical structural variables (classification of larval habitat, shading, type of water, presence of debris, current, and vegetation) in the abundance of species. GLM-normal distribution was used to examine species abundance and richness in relation to breeding site size and distance to the nearest dwelling. GLM-negative binomial was the most appropriate model for the analysis of limnological parameters to examine which ones influenced species occurrence.

The Kruskal-Wallis test was applied to the nonparametric dataset to evaluate whether differences in abundance were significant based on structural characteristics and among breeding sites and physicochemical factors of water.

Multivariate analyses (Canonical Correspondence Analysis - CCA) were used to evaluate changes in species composition and response to variations in physicochemical factors of water. Tables were constructed with values obtained with GLM for all variables tested for the collected species, with the values of the physicochemical parameters obtained in the four collections, and with species abundance per breeding site along with entomological parameters.

The data were tabulated in the software Excel-Office 2019, with support for statistical analysis. Tests and graphs were generated with the software Paleontological Statistics – Past version 1.34 (Hammer *et al.* 2001) and BioEstat version 5.0 (Ayres *et al.* 2007). The significance level for inferential and descriptive statistics was set at $\alpha = 0.05$ (95.00%).

Results

Of the 52 breeding sites identified and sampled in the District of Ilha de Santana, 50 (96.15%) were positive for *Anopheles* species. Nine species were collected, with two or more species observed in all breeding sites. *Anopheles darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* were found in 47 (94%) breeding sites, while *A. albitarsis* s.l. in 28 (56%) breeding sites.

Environmental parameters (structural characterization of larval habitats)

Of the 50 breeding sites analyzed, 45 (90%) were classified as artificial and five (10%) as natural. Three distinct types of larval habitats were identified, 45 (90%) fish farming tanks, three (6%) ponds, and two (4%) streams. By category, fish farming tanks and streams had the highest species richness, both with nine species and abundance of 1,632 and 44 specimens, respectively. In ponds, six species were found with an abundance of 157 individuals. When analyzing each breeding site individually, species richness was highest in a stream, with eight species, while the highest abundance was found in a pond, with 121 specimens. High species richness and abundance was also observed in fish farming tanks, with up to seven species and 101 individuals, respectively. All larval habitats found in the District of Ilha de Santana were classified as permanent. The two breeding sites negative for *Anopheles* larvae were a fish farming tank and a stream. The former exhibited structural characteristics similar to the others fish farming tanks and the stream showed differences regarding the nature of the water (clear) and the type of current (moderate).

The mean size of fish farming tanks was 44.43 m^2 (ranging from 25 m^2 minimum to 100 m^2 maximum). The mean size of ponds was 73.33 m^2 (60 m^2 minimum to 80 m^2 maximum) and streams, 30 m^2 (25m^2 minimum to 35 m^2 maximum) (study area defined

for our study). The mean distance from breeding sites to dwellings was approximately 42.96 m^2 (25 m^2 minimum and 75 m^2 maximum distance), with 20 (40%) breeding sites at a distance between 25 m^2 and 35 m^2 from dwellings, 19 (38,00%) between 40 m^2 and 50 m^2, 11 (22%) between 55 m^2 and 75 m^2.

The GLM-normal distribution revealed a significant negative relationship regarding abundance of *A. darlingi* (G = 324.870; β = -0.017; $p < 0.001$), *A. konderi* (G = 16.679; β = -0.030; $p < 0.001$), *A. nuneztovari* s.l. (G = 251.920; β = -0.027; $p < 0.001$), and *A. triannulatus* (G = 45.844; β = -0.012; $p < 0.001$) and the distance from breeding site to the nearest dwelling. Similarly, when analyzing species abundance and breeding site size, a negative relationship was observed for: *A. intermedius* (G = 4.186; β = -0.332; $p = 0.003$), *A. konderi* (G = 35.830; β = -0.036; $p < 0.001$), and *A. mattogrossensis* (G = 7.391; β = -1.846; $p = 0.006$). However, for *A. braziliensis* (G = 4.280; β = 0.011; $p < 0.001$), *A. darlingi* (G = 60.815; β = 0.004; $p < 0.001$), *A. nuneztovari* s.l. (G = 9.478; β = 0.003; $p = 0.002$), and *A. triannulatus* (G = 4.146; β = 0.002; $p = 0.041$), a positive relationship was found between abundance and size. Considering species richness, a negative relationship was obtained only for the variable distance and the nearest dwelling (G = 11.994; β = -0.008; $p< 0.001$).

The GML-multinomial logistic regression revealed a significant effect of the variables type of breeding site (G = 5.256; β = -1.508; $p = 0.021$), shading (G = 4.379; β = -16.679; $p = 0.036$), and especially type of water (G = 9.075; β = 1.571; $p = 0.002$) on *A. darlingi*. Abundance of *A. nuneztovari* s.l. was influenced by the variables type of breeding site (G = 5.256; β = -1.508; $p = 0.021$), shading (G = 4.379; β = -16.679; $p = 0.036$), and current (G = 8.149; β = -1.545; $p = 0.004$). *A. triannulatus* was affected by the environmental variables shading (G = 4.379; β = -16.679; $p = 0.036$), type of water

(G = 4.026; β = 0.985; p = 0.044), and the presence of debris (G = 5.723; β = -0.721; p = 0.016). *Anopheles albitarsis* s.l. was affected only by type of water (G = 6.066; β = 0.818; p = 0.013) and *A. intermedius,* by the type of breeding site (G = 5.256; β = -1.508; p = 0.021) (Table 1S, supplementary materials). According to the Kruskal-Wallis test, the highest abundance of anophelines was found in breeding sites with shading greater than 50%, polluted or murky waters with debris, no current and floating vegetation ($p < 0.001$).

Limnological parameters

During the four water collections carried out in the 20 breeding sites selected, 80 samples were obtained and analyzed for eleven physicochemical parameters. According to the CONAMA Environmental Resolution #357/2005, which establish standards for the maintenance of aquatic life in natural water bodies, of nine parameters analyzed (reference values for temperature and electrical conductivity are not established by CONAMA), only four (44.45%) were within reference standards (ammonia, nitrate, nitrite, and total dissolved solids), three (33.33%) (pH, color, and turbidity) were partially in accordance (only for some breeding sites), and two (22.22%) (dissolved oxygen and phosphate) were out of range (Table 2S, supplementary materials).

When including all breeding sites sampled, the mean water temperature was 28.03º C, with a minimum of 26.00º C and maximum of 31.10º C, with pH ranging between 5.00 and 7.30. However, immature *Anopheles* were not found in breeding sites with pH between 5.00 and 5.91 (Table 2S, supplementary materials). No significant differences were found in limnological parameters among the breeding sites analyzed (H = 1.531; p = 0.922).

Figure 2 shows the contribution of eleven limnological variables regarding the distribution of the three most abundant species collected during the four-water collection.

The first axis of the CCA explained between 65.46% and 81.05% of the total variance in species distribution and abundance in breeding sites. The second axis explained between 18.95% and 34.54%. Considering all variables analyzed in the four collections, *A. darlingi* was the most affected by pH, total dissolved solids, electrical conductivity, and nitrate. For *A. nuneztovari* s.l., the variables that most contributed were pH, dissolved oxygen, phosphate, turbidity, and color, while for *A. triannulatus,* temperature and dissolved oxygen.

The negative binomial regression revealed significant relationships between species abundance and limnological variables. A significant and positive trend was found for *Anopheles darlingi* and pH (G = 5.685; β = 3.733; p = 0.017) and ammonia (G = 5.921; β = 6.742; p = 0.014), and for *Anopheles triannulatus* and pH (G = 5.453; β = 1.093; p = 0.019), temperature (G = 5.120; β = 0.518; p = 0.023), and ammonia (G =4.957; β = 2.491; p = 0.025). For *A. braziliensis,* a significant and negative relationship was observed with nitrate (G = 3.972; β = -2.910; p = 0.046), but positive with turbidity (G = 4.052; β = 0.031; p = 0.044) (Table 1S, supplementary materials).

Entomological parameters

The Larvae Index per Man/Hour (LIMH) was higher for *A. darlingi* (0.196) and *A. nuneztovari* s.l. (0.116). Regarding breeding sites, the highest LIMH was obtained for a fish farming tank (0.776) and the lowest for a pond (0.032). A high positivity rate was found for *Anopheles darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* (0.904). The ratio of water collections used by anopheline species as breeding sites in the study area was 0.048. Despite the low absolute breeding index obtained for *A. darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* (ABI = 0.032), the relative ratio of breeding sites occupied by

these species was high as well as their abundance in breeding sites in relation to the number of water collections (RBI = 0.940) (Table 1).

Discussion

In this study, of the three types of larval habitats identified in the district of Ilha de Santana, fish farming tanks were the most productive for the development of *Anopheles* larvae with high species richness and abundance. Our GLMs analyses confirmed that fish farming tanks affect the occurrence of mainly *A. darlingi* and *A. nuneztovari* s.l., as observed in previous studies to *A. darlingi* for Rodrigues *et al.* 2008 and Saraiva *et al.* 2009. This type of habitats was the only breeding site positive throughout the year. Notably, this habitat is the main breeding site for immatures of *Anopheles* and responsible for the maintenance of the primary vector in the district, *A. darlingi*. Barros and Honório (2015), in the state of Roraima, found a significantly higher mean number of larvae per site considering all fish farming tanks sampled compared to streams. In endemic areas of malaria in the states of Acre and Amazonas, fish farming tanks were infested with immature anophelines, with on average up to four times more than natural breeding sites (Rodrigues *et al.* 2008; Reis *et al.* 2015). In recent years, several authors (Saraiva *et al.* 2009; Maheu-Giroux *et al.* 2010; Reis *et al.* 2015) warned about the risk of increase in the number of malaria cases associated with fish farming tanks, describing them as potential and preferred breeding sites for several species of *Anopheles*, especially for *A. darlingi*, contributing to the continuous transmission of this disease in the Amazonian region (Rodrigues *et al.* 2008; Saraiva *et al.* 2009; Vittor *et al.* 2009; Maheu-Giroux *et al.* 2010).

The results of GLM models also revealed a negative and statistically significant relationship between the occurrence of *A. darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* and the variable distance of breeding sites to the nearest dwelling, and a significantly positive relationship with size of the breeding site. In fact, *A. darlingi* is the most anthropophagic and synanthropic species and is considered the main vector of malaria in the Brazilian Amazon (Santos *et al.* 2009). *A. nuneztovari* s.l. and *A. triannulatus* s.l., on the other hand, exhibit wide behavioral variation in Venezuela, Colombia, Peru, and Brazil, reflecting the existence of species complexes playing the role of secondary vectors in some regions (Schoeler *et al.* 2003; Galardo *et al.* 2007; Turell *et al.* 2008; Rezende *et al.* 2009; Sinka *et al.* 2010; Rosero *et al.* 2013; Naranjo-Díaz *et al.* 2016). In the present study, although these species were found in high numbers and were associated with the shortest distance from dwellings, it should be emphasized that most fish farming tanks are distant from urban areas, where concentration of dwellings is higher. Instead, they are in transition (low concentration of dwellings) and rural areas (dwellings distant from each other - family farms), exposing their residents to a higher risk of infection with *Plasmodium.* Approximately 25% of emerging mosquitoes are estimated to have a dispersion radius of up to 450 m (Barros and Honório 2015). The maximum distance from fish farming tanks to the nearest residence in transition and rural areas was 75 m, thus very close to the dwellings and within the radius of flight dispersion of mosquitoes.

In Porto Velho, state of Rondônia, malaria was also correlated with distances from the nearest dwellings, but up to 400 m from access points as defined by the authors (proximity to the edge of the forest). These authors also recommend that houses should not be located less than 400–500 m from breeding sites (Barros and Honório 2015). Reis *et al.* (2015) found the highest abundance of *A. darlingi* up to 100 m from dwellings, and

the absence of malaria cases were reported only starting at 900 m from fish farming tanks. These authors also indicated greater exposure of residents to the malaria vector compared to residents living further away.

In this study, *A. darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* were found in 47 breeding sites, revealing a harmonious coexistence during the larval stage, although *A. darlingi* and *A. nuneztovari* s.l. were not found coexisting in the adult stage (unpublished data, Barbosa and Scarpassa). A statistically negative relationship was also observed between richness and the variable distance from breeding sites to the nearest dwelling, which is the more distant the breeding ground from the dwellings, the greater the species richness, confirming the more wild behavior of *A. intermedius*, *A. mattogrossensis*, and *A. peryassui*, as already reported by Barbosa *et al.* (2014); and the level of synanthropy of some species (*A. darlingi* and *A. albitarsis* s.l.), as described by Gomes *et al.* (2008). On the other hand, in this study we believe that *A. nuneztovari* s.l. and *A. triannulatus* may be adapting to more anthropogenic environments because they were found in great abundance and closer to the dwellings.

This study also shows the favorable conditions mainly for *A. darlingi* and *A. nuneztovari* s.l. in fish farming tanks with following characteristics: shading higher than 50%, polluted or murky waters, presence of debris, absence of current and floating vegetation, as shown by the Kruskal-Wallis test. In addition to the type of breeding site, shading was essential for maintaining abundance. Relationship between *Anopheles darlingi* and type of water, and *A. nuneztovari* s.l. and current were also observed. The multinomial logistic regression revealed the influence of areas with low light for *A. darlingi*, *A. nuneztovari* s.l., and *A. triannulatus*. However, Barros and Honório (2015) pointed out that the preference for low luminosity and proximity to humans may be

considered secondary factors, attributing the absence of water current as the fundamental parameter of the type of ecotone examined in their study. Running water in streams, characterized by turbulent flow and strong currents generated by intense rains, more pronounced during the rainy season, can induce larval mortality (Barros and Honório 2015). Water accumulation with more stable features throughout the year is found in fish farming tanks, making them more viable for the development of immatures. *Anopheles* adults do not usually lay their eggs in waters with strong currents, such as in some streams or rivers, as their larvae are not adapted to strong ripples (Williams and Pinto 2012).

Our results confirm the occurrence *A. darlingi* larvae preferably in areas with low sun exposure (shaded). The preference for shaded areas has been suggested as a behavioral characteristic of females for oviposition adapted to forest environments and essential for the proliferation of *A. darlingi* in habitats such as fish farming tanks near dwellings (Barros *et al.* 2011; Barros and Honório 2015). It should be pointed out that breeding sites characterized as polluted did not contain waste and were classified based on the dark colored water, as it could not be defined as clear water or murky. In Porto Velho, the neighborhoods with the highest API values had the highest number of breeding sites, consequently with a high density of larvae (Martins 2010). These breeding sites, within less than 20 m of distance from dwellings, had structural characteristics such as shaded margins, cloudy water, submerged vegetation and were classified as permanent breeding sites, similar to our findings (Martins 2010). In Yanomami villages, the authors reported positive associations of *A. darlingi* and *A. triannulatus* with permanent water bodies and with the presence of aquatic vegetation (submerged macrophytes) (Sánchez-Ribas *et al.* 2017).

A few studies have been conducted on the bionomy of *A. mattogrossensis*. This species belongs to the subgenus *Anopheles* and in this study, its abundance was low, indicating a preference for breeding sites with characteristics distinct from those obtained for the species of the subgenus *Nyssorhynchus,* or a low adaptability for larval habitats closer to dwellings. Although the environmental variables were not significantly associated with the abundance of *A. mattogrossensis* and the occurrence of this species was only observed in three breeding sites, it should be pointed out that most specimens were collected in a stream with moderate current, confirming its preference for more natural habitats (Tadei and Thatcher 2000).

In this study, based on limnological parameters, the analyzed habitats did not present favorable characteristics for the maintenance of aquatic life in natural water bodies. Out of the nine parameters with reference values established by CONAMA, only four were within ranges, thus unfavorable to natural predators of anopheline larvae and other Culicidae species.

According to CCA, pH was one of the limnological variables that most contribute to the abundance of *A. darlingi*, with ideal levels for the maintenance of aquatic life between 6.0 and 9.0. Values outside this range are lethal or harmful to most aquatic organisms (Cardoso 2007). The GLM-negative binomial regression confirmed the influence of this variable, revealing that *A. darlingi* is best adapted to higher pH values, within an optimal range for its survival. In general, the pH varied from slightly acidic to neutral, possibly due to soil characteristics, presence of organic matter, and rainfall (Cunha *et al.* 2003). The pH reduction in the breeding sites influences the number of species and biomass (Dantas 2011). Regardless of the type of breeding site, pH becomes

a limiting factor for the occurrence of *Anopheles* species, as no specimens were found in all breeding sites with pH below 5.91, similar to the reported by Barros (2012).

In this study, total dissolved solids also affected the abundance *A. darlingi* and the values obtained in the analyses for this parameter were all in accordance with CONAMA. This indicated excellent environmental aquatic conditions, as values above upper limits might be due to recent organic contamination with domestic, industrial, or solid waste caused by erosion on the margins of water collections, compromising aquatic life (Androeli *et al.* 2013). Another parameter shown to influence the abundance of *A. darlingi* was nitrate, which was also in line with the values established by CONAMA, as this macronutrient is essential for algal growth (Mota 2012). At low concentrations, it is a limiting factor for the development of aquatic life, while high quantities cause eutrophication (Sperling 2005). The controlled growth of aquatic plants creates environmental conditions favorable for the development of *Anopheles* larvae.

Similarly, for *A. nuneztovari* s.l. in this study, pH affected abundance, as well as dissolved oxygen, phosphate, turbidity, and color, although results for the negative binomial regression were not significant. However, these parameters limit aquatic life and affected this species, according to CCA, as in at least one of the collections, as the values of dissolved oxygen were below those established by CONAMA. Although *Anopheles* immatures breathe atmospheric oxygen, the distribution of oxygen in the water body influences the solubility of inorganic nutrients, essential for the development and survival of *Anopheles* larvae (Dantas 2011). This parameter might be the most important in water quality, as it is essential for the maintenance of aerobic and facultative aquatic organisms, taking part of several processes of the biogeochemical cycle of carbon, nitrogen, and sulfur (Cunha *et al.* 2003). Low dissolved oxygen values were obtained possibly due to

high temperatures, since the higher the ambient temperature, the lower the dissolved oxygen saturation levels in the water.

Although most breeding sites analyzed (95%) had high phosphate levels, favoring the growth of lethal bacteria to immatures, based on CCA, this parameter influenced only *A. nuneztovari* s.l., but GLM did not reveal a statistically significant relationship. Thus, phosphate was not a limiting factor for the occurrence and abundance of *A. darlingi*, *A. nuneztovari* s.l., and *A. triannulatus*. Phosphate is one of the main macronutrients for aquatic biological processes and indispensable for algal growth but can cause eutrophication at high levels (Sperling 2005). Turbidity was another parameter influencing the abundance of *A. nuneztovari* s.l., but in most breeding sites (95%), levels were within those established by CONAMA. Unlike turbidity, in most breeding sites (92.25%), color was not within established values. Color is usually associated with the presence of dissolved solids and colloidal particles, derived from the decomposition of organic matter, iron, and manganese, as well as industrial waste and domestic sewage (Sperling 2005). This may explain the dark appearance of the waters of the breeding sites surveyed. *Anopheles nuneztovari* s.l. and *A. triannulatus* were the only species influenced by this variable, as GLMs revealed a positive but not statistically significant relationship. Once again, A. *darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* seemed to be more resistant to excess organic matter in the water.

For *A. triannulatus,* the parameters that contributed the most to its abundance were nitrite, temperature, dissolved oxygen, total dissolved solids, conductivity, turbidity, and color. However, the negative binomial regression showed significant and positive correlations only with pH, temperature, and ammonia. This suggests that *A. triannulatus* can occur in aquatic environments with higher temperatures and pH. Water temperature

influences the time required for larval development, which is shorter in warmer waters (Williams and Pinto 2012).

A negative relationship was observed between *Anopheles braziliensis* nitrate and turbidity. In low concentrations, nitrate is a limiting factor for the development of aquatic life. On the other hand, the absence of particles that determine turbidity allows light rays to penetrate in water bodies, influencing the photosynthesis and growth of aquatic plants and plankton (Di Bernardo and Dantas 2005). Thus, *A. braziliensis* was sensitive to environments with excess aquatic vegetation, and preferred breeding sites with less murky waters, indicating that it is a more selective species regarding habitat.

In general, *Anopheles* species are sensitive to pollution, although CCA revealed the influence of conductivity on *A. darlingi* and *A. triannulatus*, but GLM did not reveal a significant relationship. Again, *A. darlingi* and *A. triannulatus* demonstrated greater tolerance to waters with higher concentration of pollutants and minerals, since the increase of conductivity releases of ions through the decomposition process (CETESB 2012) and can cause larval mortality.

Immatures feed mainly on debris and microalgae present in breeding sites, and these algae act as nutritional support of larvae as well as in the oxygenation of the aquatic environment (Arcos *et al.* 2018). Imbalances in the aquatic environment can trigger eutrophication and consequently result in larval mortality. This unbalance is caused by changes in physicochemical parameters: phosphorus and nitrogen forms (ammonia, nitrate, and nitrite). Some of the parameters analyzed were not within the values established by CONAMA but were still favorable for the development of immatures of some species of *Anopheles.* Other parameters have been shown to influence positively

(pH, ammonia, temperature, and turbidity) and only one negatively (nitrate) the occurrence and abundance of species.

The findings of this study confirm the results observed in previous studies (Barros and Honório 2015; Reis *et al.* 2015; Barros *et al.* 2020) that fish farming tanks play a role as permanent breeding sites with high potential for the development of immature stages of *Anopheles*, especially *A. darlingi*, in the district of Ilha de Santana. This is supported by the higher LIMH observed for this type of breeding site. Values similar to those obtained the present study were reported in São José de Ribamar, in the state of Maranhão (0.64), although Barros *et al.* (2020) found only 75 *Anopheles* larvae in this locality. In this study, *Anopheles darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* were the species that best adapted to fish farming tanks and maintained a very high positivity index throughout the study. Despite their high abundance, these species presented a low absolute index (ABI) of occupied breeding sites, considering the total of water collections. However, based on relative breeding index (RBI), *A. darlingi*, *A. nuneztovari* s.l., and *A. triannulatus* were found in almost all breeding sites, with very high values, revealing the wide distribution of these species and proving to be more tolerant to water collections with physicochemical quantities above that established for the maintenance of aquatic life. According to the GBI obtained, *Anopheles* larvae require a low water volume to develop naturally, if the environmental conditions are favorable. The volume of water and the size of the breeding area were important for the colonization of *Anopheles* immatures, as already observed by Dantas (2011) with other culicids. In inspections carried out in fish farming tanks in the east and west areas of Manaus to assess the density of larvae based on LIMH, all 141 tanks monitored were positive for *Anopheles* immatures, and only *A. darlingi* had a positivity index of 75%. These authors indicated

the importance of studies on the control of *Anopheles* larvae in artificial breeding sites (fish farming tanks and fish ponds) in the Amazon (Rodrigues *et al.* 2008). In Boa Vista, Roraima, a high positivity rate (85.70%) was also found for *A. darlingi* larvae (Barros and Honório 2015), corroborating our results (90.40%) for *A. darlingi*, *A. nuneztovari* s.l. and *A. triannulatus*.

Thus, considering all the parameters examined, the following entomological indicators were found: 1) fish farming tanks were the most significant breeding sites for the distribution and abundance of the species; 2) larval density was higher for *A. darlingi* and *A. nuneztovari* s.l.; 3) the highest positivity index in breeding sites was found for *A. darlingi*, *A. nuneztovari* s.l., and *A. triannulatus*; 4) These three species were also the most widely distributed and more resistant to water collections with physicochemical parameters above those established for the maintenance of aquatic life and that can cause eutrophication; 5) *Anopheles* larvae use a low ratio of water collections to develop.

In general, our findings indicate that larval abundance was not affected by vegetation (environmental parameters), dissolved oxygen, color, phosphate, total dissolved solids, electrical conductivity and nitrite (limnological parameters). However, the classification of the breeding site, shading, type of water, debris, current, pH, temperature, nitrate, ammonia, turbidity were determinant for some species, as discussed extensively above. Of all the parameters analyzed, shading was one of the main determinants for *A. darlingi*. Like the reported by Sánches-Ribas *et al.* (2017), water current was not a predictive factor for this species. However, our findings suggest the association of this species with waters with less disturbance, as few specimens were collected in the streams with moderate current, despite environmental and limnological

characteristics similar to those of breeding sites in which *A. darlingi* was found in high abundance.

Our findings demonstrated that malaria outbreaks in the district of Ilha de Santana will continue to occur, given the high density of the main vectors and the number of fish farming tanks in the area near human dwellings and the environmental and limnological conditions that favor their development (*A. darlingi*). In this study, several entomological analyses were performed to estimate the occupation of breeding sites by immature mosquitoes providing information on bioecology and the various factors involved in malaria transmission, such as the amount of water collections necessary for the development of immatures.

In conclusion, our findings suggest an important role of fishponds in the maintenance in high density vector populations throughout year in the studied area. Hence, we suggested that the implementation of targeted control measures and planning of management practices for fish farming tanks which will need to be routinely incorporated in malaria integrated control to reduce transmission in the Ilha de Santana.

This set of methods could be applied to reduce the number of vectors reaching adulthood, such as the application of chemical insecticides, biological agents, larvivorous fish, oils that form a film on water that prevents larvae and pupae from breathing, use of insect growth regulators that stops larva development from reaching the adult stage and the manipulation or physical elimination of larval habitats to prevent mosquito reproduction (Williams and Pinto 2012). The local community should also be assisted regarding the need for fish farming tanks and the risks involved. Alternatively, a more drastic but effective measure would be to drain and fill fish farming tanks, because during this study, all were not being used for the purpose they were built.

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Table 1S, supplementary file. Generalized Linear Models of environmental and limnological parameters of breeding sites in relation to *Anopheles* species collected in the district of Ilha de Santana, Amapá, Brazil.

Parameters	Species	GLM multinomial logistics			
		β	**SE**	***G value***	***p value***
Classification (fish farming ponds, lagoons and streams)	***A. albitarsis* s.l.**	-0.058	0.549	0.011	0.915
	A. braziliensis	-0.467	0.584	0.684	0.408
	A. darlingi	-1.508	0.642	5.256	0.021*
	A. intermedius	1.358	0.636	5.346	0.020*
	A. konderi	-0.693	0.670	1.282	0.257
	A. mattogrossensis	1.112	0.733	1.897	0.168
	***A. nuneztovari* s.l.**	-1.508	0.642	5.256	0.021*
	A. peryassui	0.771	0.566	1.89	0.169
	A. triannulatus	-0.679	0.681	0.848	0.356
Shading	***A. albitarsis* s.l.**	0.030	0.632	0.002	0.961
	A. braziliensis	-0.393	0.630	0.392	0.531
	A. darlingi	-16.679	1,519.700	4.379	0.036*
	A. intermedius	-0.405	0.712	0.316	0.574
	A. konderi	0.076	0.630	0.014	0.903
	A. mattogrossensis	-0.325	1.266	0.063	0.801
	***A. nuneztovari* s.l.**	-16.679	1,519.700	4.379	0.036*
	A. peryassui	-0.441	0.668	0.429	0.512
	A. triannulatus	-16.679	1,519.70	4.379	0.036*
Water nature	***A. albitarsis* s.l.**	0.818	0.358	6.066	0.013*
	A. braziliensis	0.289	0.315	0.862	0.353
	A. darlingi	1.571	0.595	9.075	0.002*
	A. intermedius	0.332	0.397	0.746	0.387
	A. konderi	-0.348	0.317	1.236	0.266
	A. mattogrossensis	0.929	0.909	1.306	0.253
	***A. nuneztovari* s.l.**	0.267	0.494	0.282	0.595
	A. peryassui	-0.351	0.334	1.100	0.294
	A. triannulatus	0.985	0.503	4.026	0.044*
Debris	***A. albitarsis* s.l.**	-0.172	0.219	0.627	0.428
	A. braziliensis	-0.268	0.233	1.435	0.230
	A. darlingi	-0.277	0.304	0.751	0.386
	A. intermedius	-0.482	0.356	2.336	0.126
	A. konderi	-0.177	0.228	0.633	0.426
	A. mattogrossensis	-0.221	0.556	0.179	0.671
	***A. nuneztovari* s.l.**	-0.453	0.292	2.216	0.136
	A. peryassui	-0.140	0.255	0.318	0.572
	A. triannulatus	-0.721	0.312	5.723	0.016*

Flow	***A. albitarsis* s.l.**	0.215	0.457	0.228	632.000
	A. braziliensis	-0.390	0.457	0.766	0.381
	A. darlingi	-0.984	0.525	3.102	0.078
	A. intermedius	0.293	0.468	0.373	0.541
	A. konderi	-0.259	0.457	0.334	0.563
	A. mattogrossensis	0.740	0.656	1.078	0.299
	***A. nuneztovari* s.l.**	-1.545	0.545	8.149	0.004*
	A. peryassui	0.520	0.441	1.357	0.244
	A. triannulatus	-0.370	0.607	0.334	0.563
Vegetation	***A. albitarsis* s.l.**	-0.080	0.366	0.049	0.824
	A. braziliensis	-0.127	0.360	0.126	0.722
	A. darlingi	-15.528	1,125.90	3.474	0.062
	A. intermedius	0.241	0.502	0.261	0.609
	A. konderi	0.270	0.369	0.512	0.474
	A. mattogrossensis	0.022	0.778	0.001	0.976
	***A. nuneztovari* s.l.**	0.341	0.462	0.471	0.492
	A. peryassui	-0.056	0.177	0.102	0.784
	A. triannulatus	-15.528	1,258.80	2.779	0.095
Parameters	**Species**	**GLM negative binomial model**			
		β	**SE**	***G value***	***p value***
pH	***A. braziliensis***	-1.302	1.268	0.581	0.445
	A. darlingi	3.733	1.234	5.685	0.017*
	A. konderi	-0.532	1.770	0.071	0.790
	***A. nuneztovari* s.l.**	0.296	0.556	0.320	0.571
	A. triannulatus	1.093	0.513	5.453	0.019*
Temperature	***A. braziliensis***	0.573	0.543	0.774	0.378
	A. darlingi	0.631	0.703	0.534	0.464
	A. konderi	-0.472	0.474	1.551	0.213
	***A. nuneztovari* s.l.**	-0.099	0.258	0.149	0.699
	A. triannulatus	0.518	0.203	5.120	0.023*
Dissolved oxygen	***A. braziliensis***	-0.683	1.046	0.527	0.467
	A. darlingi	0.004	1.226	0.009	0.997
	A. konderi	-0.403	1.721	0.028	0.865
	***A. nuneztovari* s.l.**	-0.020	0.334	0.005	0.944
	A. triannulatus	0.493	0.349	2.559	0.109
Nitrate	***A. braziliensis***	-2.910	1.426	3.972	0.046*
	A. darlingi	-0.893	1.651	0.230	0.630
	A. konderi	-1.562	1.119	1.221	0.269
	***A. nuneztovari* s.l.**	0.102	0.775	0.017	0.894
	A. triannulatus	-0.615	0.520	0.573	0.449

Ammonia	***A. braziliensis***	4.497	3.545	1.285	0.257
	A. darlingi	6.742	2.535	5.921	0.014*
	A. konderi	2.346	4.141	0.045	0.831
	***A. nuneztovari* s.l.**	1.601	1.005	2.894	0.088
	A. triannulatus	2.491	0.997	4.957	0.025*
Turbidity	***A. braziliensis***	0.031	0.021	4.052	0.044*
	A. darlingi	0.009	0.026	0.236	0.627
	A. konderi	-0.025	0.017	1.409	0.235
	***A. nuneztovari* s.l.**	-0.001	0.005	1.254	0.965
	A. triannulatus	-0.007	0.002	0.195	0.262
Color	***A. braziliensis***	0.005	0.006	0.437	0.508
	A. darlingi	-0.006	0.004	0.838	0.359
	A. konderi	-1.096	0.016	0.251	0.616
	***A. nuneztovari* s.l.**	0.001	0.002	0.011	0.917
	A. triannulatus	0.001	0.002	0.195	0.658
Phosphate	***A. braziliensis***	5.906	4.702	1.022	0.312
	A. darlingi	7.851	5.453	2.940	0.086
	A. konderi	-1.456	2.429	0.448	0.503
	***A. nuneztovari* s.l.**	2.441	1.812	2.426	0.119
	A. triannulatus	2.324	2.292	1.170	0.191
Total dissolved solids	***A. braziliensis***	0.026	0.218	0.022	0.879
	A. darlingi	0.078	0.112	0.451	0.501
	A. konderi	-0.102	0.126	0.756	0.384
	***A. nuneztovari* s.l.**	0.002	0.043	0.002	0.963
	A. triannulatus	0.006	0.041	0.017	0.895
Eletrical conductivity	***A. braziliensis***	-0.013	0.114	0.022	0.880
	A. darlingi	0.039	0.059	0.488	0.484
	A. konderi	-0.057	0.064	1.016	0.313
	***A. nuneztovari* s.l.**	0.003	0.022	0.024	0.844
	A. triannulatus	0.019	0.023	0.560	0.454
Nitrite	***A. braziliensis***	500.000	864.520	0.389	0.532
	A. darlingi	777.780	1.420.900	393.000	0.530
	A. konderi	-	-	-	-
	***A. nuneztovari* s.l.**	-265.150	379.220	0.399	0.527
	A. triannulatus	186.270	445.180	0.202	0.653

β: estimation; SE: standard error; * significant at $p < 0.05$; (-) Nitrite values equal to 0.000 for positive breeding sites for *A. konderi*.

Table 2S, supplementary file. Analytical limnological parameters of twenty breeding sites from the four water sample collections carried out in the district of Ilha de Santana, Amapá, Brazil.

	Breeding	pH	Temperature (º C)	Nitrate (NO^{-}_{3}-N)	Ammonia (NH_3-N)	DO (mg/L)	Turbidity (NTU)	Color	Phosphate (PO_4^{-3})	TDS (ppm)	Eletrical conductivity (µS/cm)	Nitrite (NO^{-}_{2}-N)
1ª. Sample	**3**	**6.90**	29.7	**0.5**	**0.39**	1.20	**14.50**	217	0.16	**10.00**	30.0	**0.000**
	5	**6.21**	28.9	**0.5**	**0.32**	1.46	**8.21**	131	0.15	**0.00**	20.0	**0.002**
	6	**6.16**	27.8	**0.0**	**0.24**	1.05	**11.00**	159	0.43	**10.00**	30.0	**0.000**
	10	**6.28**	28.9	**0.0**	**0.46**	2.11	**23.60**	287	0.15	**20.00**	40.0	**0.000**
	14	**6.21**	29.5	**0.0**	**0.30**	1.09	**8.26**	135	0.29	**10.00**	30.0	**0.000**
	15	**6.22**	27.1	**0.0**	**0.48**	1.26	**28.80**	337	0.51	**20.00**	40.0	**0.000**
	17	**6.28**	28.3	**0.3**	**0.25**	1.11	**7.49**	127	0.28	**20.00**	40.0	**0.000**
	18	**6.25**	28.7	**2.3**	**1.70**	2.74	133.00	1305	0.66	**10.00**	30.0	**0.000**
	24	**6.25**	28.8	**0.0**	**2.20**	0.45	152.00	1640	0.70	**20.00**	40.0	**0.000**
	26	5.86	28.4	**0.6**	**0.48**	0.35	**8.32**	234	0.47	**10.00**	20.0	**0.000**
	31	5.72	27.1	**0.4**	**0.44**	0.81	**7.57**	162	0.53	**10.00**	20.0	**0.000**
	36	5.61	28.4	**4.1**	**0.15**	1.46	**2.00**	89	0.35	**40.00**	80.0	**0.003**
	39	5.79	28.8	**0.1**	**0.45**	0.84	**8.69**	262	0.38	**10.00**	30.0	**0.000**
	41	**6.00**	31.1	**0.0**	**0.62**	3.89	**40.70**	415	0.16	**10.00**	30.0	**0.000**
	42	**6.14**	30.6	**0.0**	**0.86**	1.72	**57.40**	661	0.05	**10.00**	40.0	**0.000**
	44	**6.20**	29.0	**0.1**	**0.34**	1.80	**17.30**	220	0.13	**20.00**	50.0	**0.000**
	45	**5.92**	29.2	**0.3**	**0.24**	1.21	**6.80**	91	0.06	**20.00**	40.0	**0.000**
	46	**5.95**	28.6	**0.0**	**0.55**	1.10	**5.92**	252	0.05	**20.00**	40.0	**0.000**
	48	**6.12**	28.5	**0.0**	**0.46**	0.67	**14.00**	243	0.15	**20.00**	50.0	**0.000**
	50	**6.24**	28.1	**0.0**	**0.33**	2.16	**18.90**	210	0.09	**20.00**	40.0	**0.000**
2ª. Sample	**3**	5.84	29.4	**0.0**	**0.44**	1.88	**22.40**	328	0.20	**20.00**	40.0	**0.000**
	5	**6.17**	28.3	**0.6**	**0.56**	1.36	**13.30**	160	0.20	**10.00**	20.0	**0.000**
	6	**6.36**	27.6	**0.2**	**0.50**	4.36	**13.90**	179	0.21	**20.00**	50.0	**0.001**
	10	**6.31**	28.0	**0.0**	**0.47**	2.01	**10.30**	129	0.21	**20.00**	50.0	**0.000**

	14	**6.46**	28.4	**0.1**	**0.51**	1.86	**30.50**	355	0.47	**20.00**	50.0	**0.000**
	15	**6.49**	26.7	**0.0**	**0.48**	1.49	**11.60**	152	0.28	**20.00**	50.0	**0.000**
	17	**6.34**	27.2	**0.2**	**0.50**	1.01	**12.10**	155	0.49	**20.00**	50.0	**0.000**
	18	**6.47**	28.3	**0.0**	**0.44**	3.75	**78.20**	808	0.82	**20.00**	50.0	**0.000**
	24	**6.36**	28.1	**0.0**	**0.49**	3.08	**72.30**	734	0.49	**20.00**	50.0	**0.000**
	26	5.86	28.4	**0.0**	**0.71**	0.19	136.00	209	0.28	**10.00**	20.0	**0.002**
	31	**6.17**	27.6	**0.2**	**0.54**	0.27	**3.79**	145	0.29	**10.00**	20.0	**0.000**
	36	5.83	27.6	**1.6**	**0.56**	1.00	113.00	**34**	0.20	**30.00**	60.0	**0.000**
	39	**7.02**	30.7	**0.0**	**0.68**	4.49	**35.60**	392	0.18	**20.00**	40.0	**0.000**
	41	**7.30**	30.1	**0.0**	**0.74**	2.97	**49.60**	509	0.30	**20.00**	40.0	**0.000**
	42	**7.09**	29.8	**0.1**	**0.76**	1.59	**6.85**	88	0.43	**10.00**	30.0	**0.000**
	44	**6.50**	28.8	**0.4**	**0.75**	1.14	**7.06**	98	0.29	**20.00**	50.0	**0.000**
	45	**6.86**	28.4	**0.0**	**0.90**	2.41	**13.08**	161	0.34	**30.00**	60.0	**0.000**
	46	**6.88**	28.6	**0.0**	**0.92**	1.74	**9.86**	208	0.31	**20.00**	50.0	**0.000**
	48	**6.96**	28.9	**0.0**	**1.05**	0.39	**21.50**	293	0.46	**30.00**	60.0	**0.000**
	50	**7.09**	28.5	**0.0**	**1.06**	2.62	**17.10**	214	0.42	**20.00**	50.0	**0.000**
	3	**6.20**	27.6	**0.2**	**0.25**	1.13	**6.39**	145	**0.01**	**20.00**	40.0	**0.000**
	5	5.60	28.1	**0.8**	**0.22**	2.28	**6.69**	**40**	0.00	**15.00**	30.0	**0.002**
	6	5.48	26.4	**0.5**	**0.54**	0.99	**5.97**	138	0.07	**5.00**	10.0	**0.000**
	10	**6.05**	26.8	**0.0**	**0.66**	0.96	**28.00**	322	0.13	**20.00**	40.0	**0.000**
	14	5.48	27.2	**0.0**	**0.32**	1.21	**57.10**	500	0.29	**10.00**	20.0	**0.000**
3ª. Sample	**15**	5.42	27.5	**0.1**	**0.20**	1.03	**13.80**	168	0.07	**10.00**	20.0	**0.000**
	17	5.60	26.8	**0.0**	**0.53**	1.10	**11.10**	142	0.06	**10.00**	20.0	**0.000**
	18	5.58	27.8	**0.0**	**0.60**	1.56	**31.40**	345	0.16	**15.00**	30.0	**0.000**
	24	**6.11**	27.2	**0.0**	**0.30**	1.96	**30.40**	366	0.15	**20.00**	40.0	**0.000**
	26	5.65	26.6	**0.4**	**0.25**	0.58	**6.65**	138	**0.02**	**10.00**	20.0	**0.000**
	31	5.57	26.0	**0.1**	**0.26**	0.52	**7.56**	159	0.43	**10.00**	20.0	**0.000**

	36	5.08	27.9	**2.8**	**0.03**	1.71	**0.10**	**2**	**0.02**	**45.00**	90.0	**0.003**
	39	5.91	26.3	**0.0**	**0.73**	0.99	**16.60**	352	0.15	**15.00**	30.0	**0.000**
	41	5.52	27.8	**0.0**	**0.42**	1.04	**23.00**	238	0.04	**15.00**	30.0	**0.000**
	42	5.80	26.8	**0.0**	**0.68**	0.46	**15.00**	382	0.14	**15.00**	30.0	**0.000**
	44	**6.13**	26.3	**0.0**	**0.48**	1.10	**19.70**	253	0.13	**25.00**	50.0	**0.000**
	45	5.74	27.1	**0.1**	**0.24**	1.20	**11.80**	134	**0.03**	**10.00**	20.0	**0.000**
	46	5.61	26.3	**0.0**	**0.31**	0.47	**12.70**	206	0.12	**15.00**	30.0	**0.000**
	48	**6.17**	26.6	**0.0**	**0.60**	0.48	**31.60**	351	0.25	**25.00**	50.0	**0.000**
	50	**6.12**	27.0	**0.0**	**0.44**	2.05	**23.80**	273	0.93	**20.00**	40.0	**0.000**
4ª. Sample	**3**	5.57	28.4	**0.7**	**0.08**	0.77	**8.76**	102	0.10	**24.80**	49.6	**0.000**
	5	5.19	27.5	**0.9**	**0.00**	0.83	**5.23**	**53**	0.10	**9.30**	18.6	**0.000**
	6	5.85	27.1	**0.0**	**0.18**	0.77	**9.47**	157	0.24	**15.60**	31.2	**0.000**
	10	**6.09**	27.3	**0.0**	**0.30**	0.80	**24.90**	277	0.20	**18.80**	37.6	**0.000**
	14	5.04	28.1	**0.7**	**0.00**	0.77	**6.54**	**62**	0.15	**9.85**	19.7	**0.001**
	15	5.81	27.9	**0.0**	**0.33**	0.80	**27.10**	203	0.13	**12.50**	25.0	**0.000**
	17	5.21	28.1	**0.0**	**0.10**	0.79	**12.60**	130	0.10	**8.00**	16.0	**0.000**
	18	5.21	29.1	**0.4**	**0.00**	0.81	**1.64**	**40**	0.08	**6.30**	12.6	**0.000**
	24	5.80	29.6	**0.0**	**0.51**	0.81	**44.50**	433	0.12	**10.85**	21.7	**0.000**
	26	5.20	27.6	**0.0**	**0.02**	0.77	**9.31**	150	0.07	**8.00**	16.0	**0.000**
	31	5.28	26.7	**0.0**	**0.15**	0.84	**21.20**	192	0.15	**10.00**	20.0	**0.000**
	36	5.33	28.4	**3.5**	**0.00**	0.76	**6.87**	**64**	0.28	**45.20**	90.4	**0.002**
	39	5.00	27.8	**0.0**	**0.40**	0.71	**28.80**	336	0.26	**9.65**	19.3	**0.000**
	41	5.62	27.5	**0.0**	**0.15**	0.79	**16.00**	188	0.20	**13.80**	27.6	**0.000**
	42	5.68	27.7	**0.0**	**0.16**	0.82	**8.62**	152	0.20	**13.15**	26.3	**0.000**
	44	5.79	28.2	**0.0**	**0.30**	0.71	**19.00**	231	0.33	**23.95**	47.9	**0.000**
	45	**6.13**	27.3	**0.0**	**0.26**	0.78	**19.00**	223	0.63	**24.85**	49.7	**0.000**
	46	**6.15**	27.0	**0.0**	**0.18**	0.80	**20.00**	208	0.24	**8.85**	17.7	**0.000**

	48	5.38	27.6	**0.0**	**0.30**	0.73	**18.00**	235	0.36	**22.20**	44.4	**0.000**
	50	**6.18**	28.7	**0.0**	**0.22**	0.79	**18.80**	226	0.17	**18.00**	36.0	**0.000**

In bold, physical-chemical parameters that were in accordance with the reference values established by Resolution #357/2005 of March 17th – CONAMA, and Resolution #430/2011 of May 13th – CONAMA.

Table 1. Number of individuals collected per species and entomological indices (LIMH, PI, GBI, ABI and RBI) in breeding sites in the district of Ilha de Santana, Amapá, Brazil.

Breeding	*A. albitarsis* s.l.	*A. braziliensis*	*A. darlingi*	*A. intermedius*	*A. konderi*	*A. mattogrossensis*	*A. nuneztovari* s.l.	*A. peryassui*	*A. triannulatus*	Total	LIMH
BS1	7	1	37	-	-	-	28	-	15	**88**	**0. 564**
BS2	10	3	40	2	1	-	16	-	21	**93**	**0. 596**
BS3	8	7	27	-	-	-	12	-	12	**66**	**0. 423**
BS4*	2	-	5	-	-	-	4	2	3	**16**	**0. 103**
BS5	9	2	25	-	-	-	17	-	13	**66**	**0. 423**
BS6*	1	-	9	2	-	-	4	-	4	**20**	**0. 128**
BS7	3	3	14	-	-	-	2	-	4	**26**	**0. 167**
BS8	-	-	7	-	-	-	5	-	6	**18**	**0. 115**
BS9	-	-	-	-	-	-	-	-	-	**-**	**0. 000**
BS10	2	-	12	-	2	-	3	-	5	**24**	**0. 154**
BS11	2	-	15	-	-	-	2	-	7	**26**	**0. 167**
BS12	3	1	5	-	5	-	7	2	3	**26**	**0. 167**
BS13	-	1	7	-	-	-	7	-	7	**22**	**0. 141**
BS14	2	-	2	1	-	-	2	-	3	**10**	**0. 064**
BS15	2	1	12	-	-	-	7	-	6	**28**	**0. 179**
BS16	2	-	4	-	-	-	3	-	4	**13**	**0. 083**
BS17	-	-	17	-	12	1	14	1	6	**51**	**0. 327**
BS18	-	-	-	-	2	-	-	1	2	**5**	**0. 032**
BS19	1	-	12	-	-	-	6	-	3	**22**	**0. 141**
BS20	-	2	6	-	-	-	8	-	3	**19**	**0. 122**
BS21	-	-	9	-	-	-	8	-	6	**23**	**0. 147**
BS22	1	-	6	1	-	1	2	-	5	**16**	**0. 103**
BS23	-	-	1	-	-	-	6	2	3	**12**	**0. 077**
BS24	1	2	11	-	2	-	14	-	11	**41**	**0. 263**

BS25	9	5	32	-	1	-	16	-	13	**76**	**0. 487**
BS26*	3	12	56	1	-	-	30	-	19	**121**	**0. 776**
BS27	2	3	16	-	4	-	8	-	2	**35**	**0. 224**
BS28	5	2	20	2	5	-	2	-	4	**40**	**0. 256**
BS29	2	-	22	-	-	-	11	-	4	**39**	**0. 250**
BS30	-	4	19	-	8	-	3	2	6	**42**	**0. 269**
BS31	-	3	10	-	-	-	2	-	-	**15**	**0. 096**
BS32	-	-	12	-	6	-	2	2	4	**26**	**0. 167**
BS33	-	1	12	-	-	-	3	-	2	**18**	**0. 115**
BS34	3	-	10	-	-	-	-	-	4	**17**	**0. 109**
BS35**	-	-	-	-	-	-	-	-	-	**-**	**0. 000**
BS36	-	2	7	-	1	-	6	-	2	**18**	**0. 115**
BS37	-	-	8	-	3	-	3	-	-	**14**	**0. 090**
BS38	1	-	17	4	7	-	18	-	19	**66**	**0. 423**
BS39	-	2	13	-	4	-	21	2	19	**61**	**0. 391**
BS40	-	1	8	-	-	-	14	-	14	**37**	**0. 237**
BS41	-	-	2	1	-	-	12	2	9	**26**	**0. 167**
BS42	4	-	11	-	3	-	2	2	15	**37**	**0. 237**
BS43**	4	2	12	2	1	5	-	2	7	**35**	**0. 224**
BS44	-	3	14	-	-	-	3	2	9	**31**	**0. 199**
BS45	6	-	20	-	4	-	21	1	8	**60**	**0. 385**
BS46	-	4	48	2	3	-	25	-	19	**101**	**0. 647**
BS47	-	2	14	-	-	-	4	-	5	**25**	**0. 160**
BS48	-	-	-	-	3	-	3	-	-	**6**	**0. 038**
BS49**	-	-	-	1	-	-	4	2	2	**9**	**0. 058**
BS50	-	2	32	-	2	-	18	-	10	**64**	**0. 410**
BS51	3	2	15	2	1	-	16	-	3	**42**	**0. 269**

BS52	3	-	19	-	5	-	10	2	2	**41**	**0. 263**
Total	**101**	**73**	**732**	**21**	**85**	**7**	**434**	**27**	**353**	**1,833**	
LIMH	**0. 027**	**0. 019**	**0. 196**	**0. 006**	**0. 023**	**0. 002**	**0. 116**	**0. 007**	**0. 094**	**0. 490**	
PI	**0. 538 (53.80%)**	**0. 500 (50.00%)**	**0.904 (90.40%)**	**0. 231 (23.10%)**	**0. 442 (44.20%)**	**0. 058 (5.80%)**	**0. 904 (90.40%)**	**0. 289 (28.90%)**	**0. 904 (90.40%)**		
GBI										**0. 048**	
ICA	**0. 019**	**0. 017**	**0. 032**	**0. 008**	**0. 015**	**0. 002**	**0. 032**	**0. 010**	**0. 032**		
RBI	**0. 560**	**0. 520**	**0. 940**	**0. 240**	**0. 460**	**0. 060**	**0. 940**	**0. 300**	**0. 940**		

* Pond; ** Stream. LIMH: Larvae Index per Man/Hour; PI: Positivity Index; ABI: Absolute Breeding Index; GBI: General Breeding Index; ABI: Absolute Breeding Index; RBI: Relative Breeding Index.

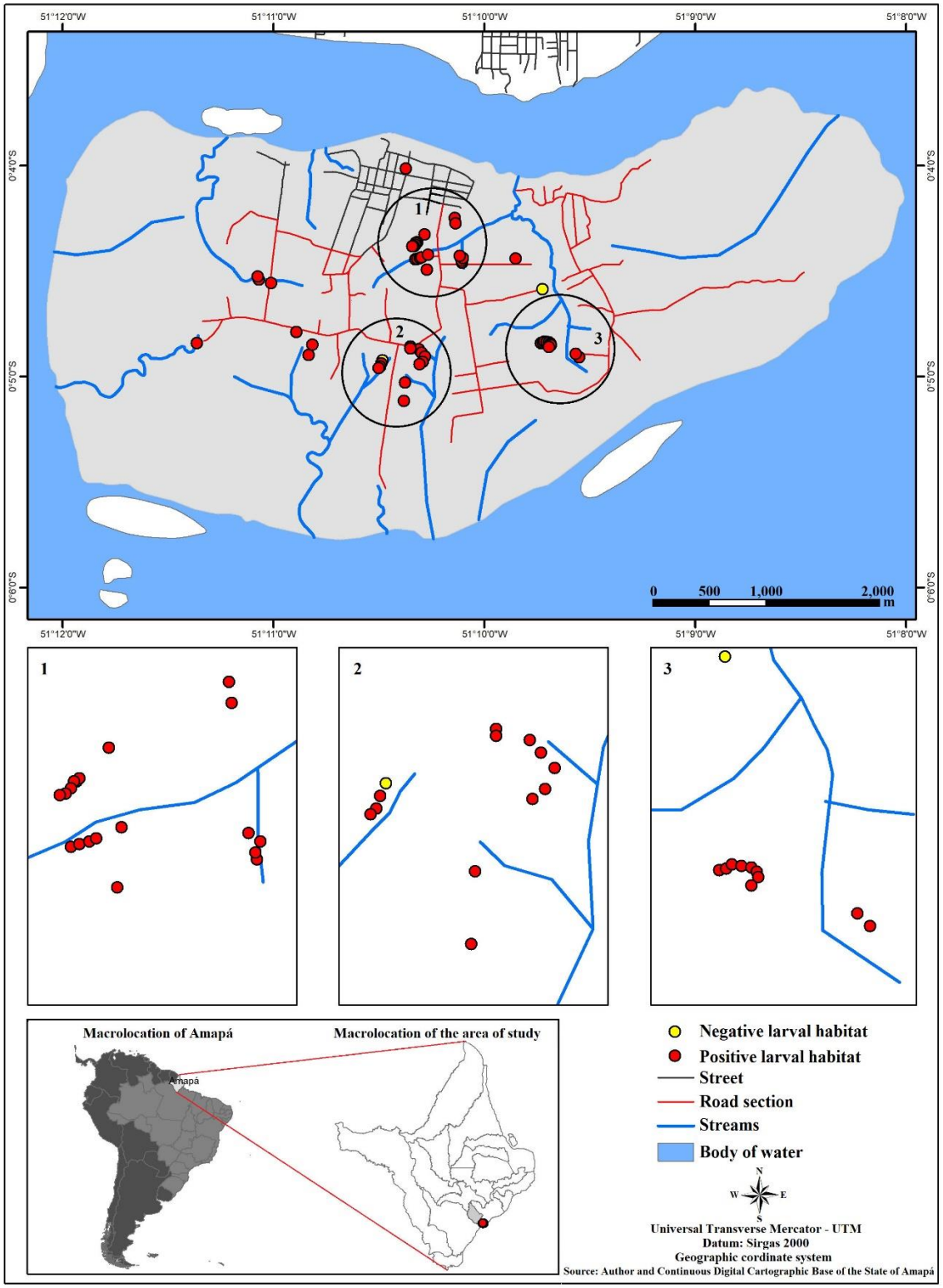

Figure 1. Hydrographic map of the District of Ilha de Santana, containing the 52 georeferenced breeding sites (immature collection points). 1, 2 and 3 - Enlarged image of the area (highest concentration of breeding sites).

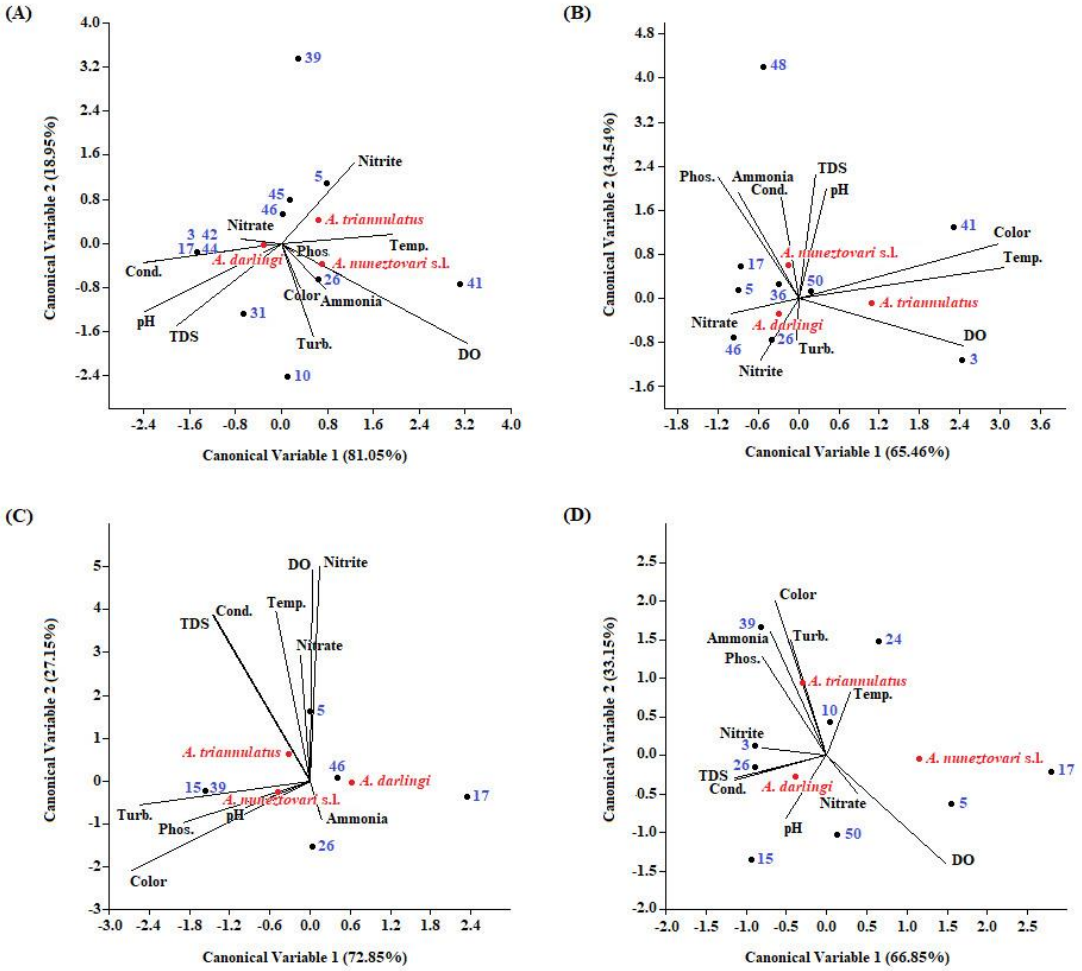

Figure 2. Canonical Correspondence Analysis (CCA), biplot of ordering diagram showing the dispersion of the three species of *Anopheles* larvae collected in greater abundance and in the largest number of breeding sites and the eleven variables of the limnological parameters contributing to the first two canonical axes; (A) Sample collected in November/2019; (B) Sample collected in December/2019; (C) Sample collected in February/2020; (D) Sample collected in June/2020. Black spots: medium centroid for each breeding site (collection sites); Red dots: medium centroid for *Anopheles* species. In brackets on the axes: contribution of each canonical variable to the total variation.

3. SÍNTESE

Anopheles darlingi foi a espécie mais abundante durante as coletas dos adultos e foi a espécie que mais contribuiu para a similaridade existente dentro de cada grupo de amostras: nas três áreas de estudo e nas duas estações. Igualmente, foi a espécie que mais contribuiu com o ambiente durante as coletas das formas imaturas. Também foi o anofelino mais frequente no intradomicílio, o mais antropofílico, apresentou as maiores atividade horária hematofágica e atração por mosquito/homem/hora nas primeiras horas com alta taxa de paridade, confirmando o início da noite como período de maior atividade desta espécie, além de ter sido a única espécie positiva para *P. vivax*, demonstrando sua importância médica e seu envolvimento na transmissão da malária humana no Distrito da Ilha de Santana. Esta espécie ainda apresentou características generalistas, provavelmente decorrente da sua ampla plasticidade adaptativa. Embora, o mapa de kernel tenha revelado três regiões críticas de alta vulnerabilidade para a transmissão da malária no distrito da Ilha de Santana, todas concentradas nas áreas de transição e rural, onde a maioria dos tanques de piscicultura estão localizados. Para as formas imaturas de *A. darlingi*, os tanques de piscicultura apresentaram maior produtividade na estação seca, quando ocorreu a maior insolação anual e o menor indice de pluviosidade, coincidindo com o aumento dos casos de malária, como demonstrado em estudos prévios. *Anopheles darlingi* também demonstrou ser mais tolerante a águas com maior concentração de poluentes e minerais, conforme demonstraram os resultados dos parâmetros limnológicos dos criadouros.

Os resultados também indicaram que *A. albitarsis* s.l. esteja contribuindo como vetor de malária neste distrito, coexistindo harmonicamente com o *A. darlingi*. Ambas as espécies foram dominantes na área urbana, revelando a vunerabilidade dos moradores daquela área à transmissão da malária.

As espécies mais abundantes, durante as coletas dos adultos e durante todo o periodo de amostragem, foram *A. darlingi*, *A. albitarsis* s.l. e *A. nuneztovari* s.l. Nas coletas dos imaturos, as espécies mais abundantes foram *A. darlingi*, *A. nuneztovari* s.l. e *A. triannulatus;* essas espécies foram as que melhor se adaptaram aos tanques de piscicultura e mantiveram um índice de positividade (IP) bastante elevado durante todo o estudo, foram também as mais amplamente distribuídas, encontradas em quase todos os criadouros (ICR) e demonstraram ser mais resistentes a coleções hídricas com grandezas físico-químicas acima do estabelecido para manutenção da vida aquática, com valores que causam a eutrofização do ambiente.

Embora *A. nuneztovari* s.l. durante as coletas dos adultos tenha sido encontrada em altas densidades, esta espécie foi coletada predominantemente no peridomicílio e apresentou comportamento zoofílico, com picos após o aumento do número de casos de malária, sugerindo

que esta espécie não está envolvida na transmissão da malária no distrito. Quando *A. darlingi* apresentou dominância, essa espécie foi encontrada em baixa densidade, e o inverso também foi observado. Também foi observada um alta dissimilaridade entre *A. nuneztovari* s.l. e *A. darlingi*, com base na análise do método Bray-Curtis. Este resultado nos permite inferir uma competição interespecífica ou impossibilidade de coexistência na fase adulta, o que não foi observado durante as coletas dos imaturos, quando foi obtido um alto índice de similaridade e afinidade entre essas espécies.

O comportamento de *A. braziliensis* foi semelhante ao de *A. albitarsis* s.l., sendo encontrado predominantemente no peridomicílio com tendências antropofílicas. Observou-se um aumento na densidade dessa espécie, com picos que antecederam o aumento dos casos de malária. Portanto, esta espécie pode estar contribuindo com a manutenção da malária na área, como um vetor secundário. Por outro lado, as formas imaturas de *A. braziliensis* demonstraram ser bastante sensível e exigente em relação a ambientes com excesso de vegetação aquática, apresentando preferência por criadouros com águas menos turvas, indicando ser uma espécie mais seletiva quanto ao habitat.

Anopheles mattogrossensis foi encontrada em baixa abundância. Na fase de larva demonstrou preferência por criadouros com características distintas das obtidas para as espécies do subgênero *Nyssorhynchus*, sendo coletado em maior parte em igarapé com correnteza moderada. Esta espécie também não apresentou afinidade com nenhum outro anofelino, quando coletada na fase adulta. Foi a única espécie estatisticamente diferente em termos de presença/ausência e abundância em relação às demais espécies capturadas, além de ter sido a única espécie considerada acidental durante as coletas de quatro horas e das formas imaturas.

Este estudo demonstrou evidências suficientes de que os tanques de piscicultura são os principais criadouros das formas imaturas dos anofelinos e são os responsáveis pela manutenção do principal vetor, *A. darlingi*, no Distrito da Ilha de Santana. Estes tanques, independente do período sazonal, continuam proporcionando condições favoráveis ao desenvolvimento dos vetores. Este tipo de criadouro apresentou condições favoráveis principalmente para o desenvolvimento de *A. darlingi* e *A. nuneztovari* s.l. Além do tipo de criadouro, outras características ambientais foram essenciais, como o sombreamento para a abundância das espécies. *Anopheles darlingi* também apresentou uma relação com a natureza da água e *A. nuneztovari* s.l. com a correnteza, além da preferência por baixa luminosidade para *A. darlingi*, *A. nuneztovari* s.l. e *A. triannulatus*.

Surtos de malária no Distrito da Ilha de Santana continuarão a acontecer, considerando a densidade elevada do principal vetor e o número de tanques de piscicultura existentes na área

e próximos às habitações humanas, além das condições ambientais e limnológicas que favorecem o desenvolvimento dos principais vetores da malária. Reconhecemos a importância dos métodos de controle atuais, mas recomendamos estratégias complementares adaptadas às condições regionais / locais, dadas as características inerentes de *A. darlingi*, sua alta capacidade adaptativa em ambientes que sofrem processo de antropização, bem como sua plasticidade e heterogeneidade comportamental.

Portanto, recomendamos um sistema de manejo integrado que atenda a um conjunto de métodos aplicados com intuito de reduzir o número de vetores a atingirem a fase adulta, tais como aplicação de inseticidas químicos, agentes biológicos, peixes larvívoros, aplicação de óleos que formam uma película sobre a água impedindo que as larvas e pupas respirem, uso de reguladores do crescimento dos insetos, que impedem as larvas de se desenvolverem até à fase adulta e a manipulação ou eliminação física de habitats larvares para impedir a reprodução do mosquito. Sugerimos também o acompanhamento junto à comunidade local para avaliar a necessidade da permanência dos tanques de piscicultura com exclarecimentos dos riscos. Alternativamente, uma medida mais drástica, mas eficaz, seria a drenagem e aterro desses tanques, pois observamos durante a realização deste estudo que todos estavam inoperantes para o propósito que foram construídos.

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