Acta Tropica 213 (2021) 105751

Contents lists available at ScienceDirect

Acta Tropica

journal homepage: www.elsevier.com/locate/actatropica

Host feeding patterns of *Nyssorhynchus darlingi* (Diptera: Culicidae) in the Brazilian Amazon

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ARTICLE INFO

Keywords:
Human blood index
blood-feeding behavior
rural communities
malaria
Amazon

ABSTRACT

Nyssorhynchus darlingi (Root) is the dominant malaria vector in the Brazilian Amazon River basin, with additional Anophelinae Grassi species involved in local and regional transmission. Mosquito blood-feeding behavior is an essential component to define the mosquito-human contact rate and shape the transmission cycle of vector-borne diseases. However, there is little information on the host preferences and blood-feeding behavior of Anophelinae vectors in rural Amazonian landscapes. The barrier screen sampling (BSS) method was employed to sample females from 34 peridomestic habitats in 27 rural communities from 11 municipalities in the Brazilian Amazon states of Acre, Amazonas, Pará and Rondônia, from August 2015 to November 2017. *Nyssorhynchus darlingi* comprised 97.94% of the females collected resting on barrier screens, and DNA sequence comparison detected 9 vertebrate hosts species. The HBI index ranged from 0.03–1.00. Results revealed the plasticity of *Ny. darlingi* in blood-feeding on a wide range of mainly mammalian hosts. In addition, the identification of blood meal sources using silica-dried females is appropriate for studies of human malaria vectors in remote locations.

1. Introduction

Mosquito blood-feeding behavior is responsible for the spread of a myriad of pathogens, such as arboviruses, nematodes and protozoa parasites to humans (Takken and Verhulst, 2013; Reeves et al., 2018a; Holderman et al., 2018). As a key component in the dynamics of vector-borne disease transmission, mosquitoes continue to be a major focus of research to obtain new information on ecology, blood-feeding patterns and other factors involved in malaria transmission (Cohen et al., 2017; Reeves et al., 2018a; Tedrow et al., 2019; O'Donnell et al., 2019).

Depending on species and population, mosquito blood-feeding foraging behavior can range from zoophilic to opportunistic to anthropophilic. For example, *Aedes baisasi* Knight & Hull feeds exclusively on fish (Miyake et al., 2019) and *Uranotaenia sapphirina* (Osten Sacken) on annelids (Reeves et al., 2018b; Holderman et al., 2018). *Wyeomyia smithii* (Coquillett) is a Nearctic pitcher-plant mosquito that includes two contrasting populations, southern blood-feeders, and northern obligate non-biters. The southern population further encompasses avid biters and isolated non-biters. Recently, Bradshaw and collaborators compared the differential expression of genes in these populations, demonstrating that the evolution of non-biting behavior compared with biting behavior resulted in a reduction in metabolic investment, greater reliance on opportunistic metabolic pathways, and less reliance on olfactory sensory input compared with visual (Bradshaw et al., 2018).

Foraging behavior of blood-feeding Anophelinae Grassi mosquitoes includes multiple feeds on same host species (cryptic) or different host species (patent) within the same gonotrophic cycle, a behavior that can enhance *Plasmodium* transmission (Tedrow et al., 2019). Patent blood-feeding has been described in *Anopheles sacharovi* Favre (Boreham and Garret-Jones, 1973), *Nyssorhynchus darlingi* (Root) (Moreno et al., 2017), and recently in several species in the subgenus *Cellia* (Tedrow et al., 2019).

The blood-feeding plasticity of Anophelinae vector species can

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https://doi.org/10.1016/j.actatropica.2020.105751
Received 28 October 2020; Accepted 31 October 2020
Available online 6 November 2020
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influence transmission patterns of malaria (Benelli and Beier, 2017; Prussing et al., 2018). For example, application of DDT insecticide in the Solomon Islands in the 1970s (Russell et al., 2013) induced a behavioral shift in *Anopheles farauti* Laveran from a late-night indoor feeding phenotype to an early outdoor one, resulting in greater human-vector contact. Similar shifts have been documented post-long-lasting insecticidal net (LLINs) and indoor residual spraying (IRS) interventions in *Anopheles gambiae* Giles and *Anopheles arabiensis* Patton (Gatton et al., 2013; Takken, 2002; Ferguson et al., 2010), and *Ny. darlingi* (Prussing et al., 2018). LLINs can also influence shifts in host affinity. In Burkina Faso, malaria control with the extensive use of LLIN promoted the emergence of a zoophilic population of *An. gambiae* s.s. that shifted from feeding predominantly on humans to feeding mainly on calves (Lefèvre et al., 2009).

Considering the current global focus on malaria control and *P. falciparum* Welch malaria elimination (WHO, 2017), the in-depth knowledge of field malariology and ecology (Baird, 2017), combined with novel collection methods, are increasingly necessary. The barrier screen sampling (BSS) method was initially employed in a study to evaluate blood meal, parity rate and other entomological parameters in Indonesia, the Solomon Islands, and Papua New Guinea, where the primary vectors are exophilic and rest in thick vegetation (Burkot et al., 2013). Subsequently, this method has been employed broadly: Oceania (Logue et al., 2016; Russell et al., 2016; Keven et al., 2017; Davidson et al., 2018; Pollard et al., 2019); South Central Africa, Zambia (Stevenson et al., 2016); South East Africa, Madagascar (Tedrow et al., 2019); and Iquitos, Peru (Moreno et al., 2017; Saavedra et al., 2019). In Queensland, Australia, the effectiveness of BSS was improved by the use of a multiple 3 × 3 Latin square design to reduce bias of geographic location (Pollard et al., 2019). A different variant of the BSS, the Quadrant Enabled Screen Trap, successfully collected exophilic and exophagic anophelines in the Western Highland Fringe of Madagascar (Tedrow et al., 2019).

Mosquito blood-feeding behavior is an essential component of the calculation of the mosquito-human contact rate. An important entomological metric, the human blood index (HBI), indicates the proportion of the mosquito blood meals obtained from humans (Garret-Jones, 1964). HBI was first measured in resting female *Ny. darlingi* in Amapá state, Brazil, where it ranged from 0.017 to 0.405 (Zimmerman et al., 2006). In contrast, in females collected on barrier screens in peri-Iquitos, Amazonian Peru, the HBI was markedly higher (0.58 to 0.87) (Moreno et al., 2017), and north of Iquitos, in the Mazan district, it was 0.42–0.75 (Saavedra et al., 2019). Additional metrics to quantify the preference of mosquitoes for available blood resource are the forage ratio and the selection index (Savage, 1931; Hess et al., 1968; Manly et al., 1993; Lardeux et al., 2007). The first study to quantify and analyze blood-feeding behavior in *Ny. darlingi* confirmed extensive anthropophily as well as an affinity for Galliformes over 3 years of collections (Moreno et al., 2017). A subsequent study, on different river systems north of Iquitos, supported this study by the detection of a substantial role of Galliformes in providing blood meals (Saavedra et al., 2019). These studies provide strong evidence of blood-feeding opportunism by *Ny. darlingi*.

Nyssorhynchus darlingi is the main malaria vector in the Amazon River basin (reviewed in Hiwat and Bretas, 2011). Other species such as members of the Albitarsis Complex are also involved in *Plasmodium* transmission locally and regionally (Sinka et al., 2010). As there is little information on host preferences and blood-feeding behavior of vectors in Brazilian Amazonian rural landscapes, we designed the study to determine: (1) the degree of anthropophily (HBI) in *Ny*. darlingi; (2) identification of blood feeding hosts of *Ny. darlingi* and other species of the subfamily Anophelinae in peridomestic habitats.

2. Materials and Methods

2.1. Study sites and mosquito collections

Anophelinae adults were collected using the barrier screen sampling (BSS) (Fig. 1) method from August 2015 to November 2017, during the wet-dry transition, and in the dry season, from 34 peridomestic habitat locations within 27 rural communities in 11 municipalities in the Brazilian Amazon states of Acre, Amazonas, Pará and Rondônia (Table 1; Fig. 2). BSS was constructed from a grey mesh fiberglass window screen, 2 m high and 12 m long, and screens were placed outdoors within ~ 5 m of houses, between houses and potential oviposition/resting sites to intercept mosquitoes (Fig. 1). Outdoor mosquito collections were conducted once in each of the 34 peridomestic habitats, within ~ 5 m of each house, from 18:00–22:00 h. Mosquitoes were collected using a manual aspirator by two to four collectors who were protected by clothing and hats that prevented them from being bitten by mosquitoes. Mosquitoes were aspirated from both sides of the screen for 10 minutes, and collectors moved away for 20 minutes; thus, the BSS was visited every 30 minutes. Mosquitoes were euthanized with ethyl acetate ($C_4H_8O_2$) vapors twice per hour. Samples were stored immediately in plastic containers with silica gel, separated by date, location, peridomestic habitat and hour of collection.

Laboratory procedures were conducted at Laboratório de Entomologia de Saúde Pública – Sistemática Molecular, Faculdade de Saúde Pública, Universidade de São Paulo (LESP-SM). Specimens were identified to species using the morphological identification key of Forattini (2002). The nomenclature adopted for the subfamily Anophelinae is that proposed by Foster et al. (2017) that elevated to genus level the neotropical subgenera *Kerteszia, Stethomyia, Lophopodomyia*, and *Nyssorhynchus*. Subsequently, each female was labeled and stored individually with silica gel at room temperature for analysis. Females were visually classified as blood-fed or unfed, and bisected in two, head plus thorax / abdomen, using a sterile entomological pin. Specimens were transferred to individual labeled plastic vials and stored at -80 °C until genomic DNA extraction. During maceration, the presence of a reddish solution was a second visual check of the initial blood-fed / unfed status.

2.2. Vertebrate survey in the peridomestic habitat

For each location, a survey of the presence and number of domestic and pet sylvatic animals was carried out 2 h prior to the start of sampling mosquitoes with BSS by one collector with help from the owners.

Fig. 1. Barrier made in gray fiberglass screen. Every 3 meters, a 2-meter aluminum tube was attached to keep the screen stretched and without folds that could hinder the collection of the resting mosquitoes.

Table 1
Geographical coordinates of the localities where field collections were conducted in the peridomestic habitats in rural settlements across 4 Amazonian states, Brazil. The collection code is formed by 2 letter regarding the state abbreviation, 3 letter municipality and number of peridomestic site.

Code	Locality	Longitude	Latitude	Date
AC-ACR-1	Ramal Porto Dias	−66.817	−10.010472	08/24/2015
AC-ACR-2	Ramal Porto Dias	−66.864611	−9.954333	08/29/2015
AC-ACR-3	Ramal do Granada	−67.107194	−9.776667	08/30/2015
AC-ROD-1	Gleba 13 de Maio	−72.695617	−7.789600	06/28/2017
AC-CRU-1	Ramal Buritirama	−72.709317	−7.721300	07/04/2017
AC-CRU-2	Ramal Buritirama	−72.714783	−7.688950	07/07/2017
AC-CRU-3	Vila Lagoinha	−72.486083	−7.738667	07/21/2017
AC-CRU-4	PDS Jamil Jereissati - Ramal do José Alves	−72.66567	−7.28575	07/22/2017
AM-LAB-1	Rodovia BR230 km25; PA Umari Ramal Boa Água	−64.666417	−7.386668	08/03/2015
AM-LAB-2	Rodovia BR230 km26; PA Paciá	−64.681089	−7.505199	08/06/2015
AM-LAB-3	Rodovia BR230 km20; PA Umari	−64.740907	−7.347613	08/09/2015
AM-HUM-1	Vila Cristolândia	−63.287698	−7.886163	07/16/2016
AM-HUM-2	Realidade	−63.100657	−6.984685	07/19/2016
AM-HUM-3	Realidade	−63.112093	−6.993511	07/20/2016
AM-ITA-1	Comunidade Nova Brasilia, Ramal do INCRA	−59.118226	−3.070980	11/23/2016
AM-ITA-2	Novo Remanso, Ramal do Minério	−59.190896	−2.867820	11/27/2016
AM-ITA-3	Novo Remanso, Ramal do Minério	−59.183318	−2.897243	11/22/2016
AM-ITA-4	Novo Remanso	−59.072889	−3.093556	11/26/2016
AM-GUA-1	Vila Gama	−72.644200	−7.294567	07/12/2017
AM-GUA-2	Ramal do Gama	−72.637517	−7.328283	07/14/2017
AM-GUA-3	Badejo do Meio	−72.705933	−7.474417	07/16/2017
AM-PRE-1	Vila Nova Jerusalem	−60.27009	−1.47379	08/10/2017
AM-PRE-2	Ramal do Estrela	−60.238490	−1.492790	08/13/2017
AM-PRE-3	Ramal do Osvaldo	−60.306040	−1.539620	08/16/2017
AM-PRE-4	Ramal da Morena	−59.342450	−2.107010	08/19/2017
AM-SGC-1	Comunidade Santo Antonio	−67.001667	−0.071389	11/10/2017
AM-SGC-2	Estrada Porto Camanaus	−66.958611	−0.147222	11/19/2017
AM-SGC-3	Tiago Montalvo	−67.099167	−0.113056	11/14/2017
AM-SGC-4	Miguel Quirino	−67.073889	−0.119167	11/16/2017
PA-PAC-1	PA Cururuí, PAD Núcleo G	−50.298032	−3.627461	04/23/2016
PA-PAC-2	PA Cururuí, Invasão	−50.219966	−3.479302	04/26/2016
RO-MCH-1	Belo Horizonte, Galo Velho, linha 14	−62.23746	−9.1773	10/20/2015
RO-MCH-2	Belo Horizonte, Galo Velho, linha 10	−62.255212	−9.223237	10/25/2015
RO-MCH-3	Belo Horizonte, Galo Velho, linha 9	−62.286399	−9.222947	10/27/2015

States (AC = Acre; AM = Amazonas; PA = Pará; RO = Rondônia)
Municipalities (ACR = Acrelândia; ROD = Rodrigues Alves; CRU = Cruzeiro do Sul; LAB = Lábrea; HUM = Humaitá; ITA = Itacoatiara; GUA = Guajará; PRE = Presidente Figueiredo; SGC = São Gabriel da Cachoeira; PAC = Pacajá; MCH = Machadinho d'Oeste)

2.3. Genomic DNA extraction and blood source identification

Genomic DNA from abdomens was extracted from individual specimens using the salting-out method by Miller et al. (1998). Vertebrate blood DNA from the following potential hosts was used as positive controls: yacare, Genus *Caiman*; bat, Order Chiroptera; cat, *Felis silvestris catus*; chicken, *Gallus gallus*; cow, *Bos taurus*; dog, *Canis lupus familiaris*; duck, Family Anatidade; horse, *Equus caballus*; monkey, *Sapajus xanthosternos*; and human, *Homo sapiens*.

To identify the blood meal source, fragments of mitochondrial gene cytochrome b (*Cytb*), cytochrome oxidase subunit I (*COI*) from genomic DNA, and *16S* ribosomal (*16S*) DNA were used. Vertebrate DNA was PCR amplified using primers in Table 2. PCR was carried out in a final volume of 25 μL, containing 9.5 μL of ddH_2O, 12.5 μL of GoTaq Master Mix, 1 μL of each primer and 1 μL of DNA template. Thermal cycling conditions for *Cytb* and *COI* primers were: initial denaturation for 15 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 sec, annealing at 50 °C for 30 sec and extension at 72 °C for 60 sec followed by a final extension at 72 °C for 7 min (Townzen et al., 2008). The thermal cycling conditions used for 16S were as follows: 95°C for 5 min of enzyme activation followed by 40 cycles of 95°C for 12 sec, 59°C for 30 sec and 70°C for 25 sec, and 1 cycle for final extension of 7 min at 70°C (Schnell et al., 2018). PCR amplicons were visualized on a 1.5% agarose gel stained with GelRed (Biotium, Hayward, CA).

The PCR amplicons were purified using PEG / NaCl solution (20% polyethylene glycol 800 / 2.5 M NaCl) precipitation protocol and run on agarose gel to verify amplification. Sanger sequencing reactions were undertaken with the same sets of primers (Table 2), and BigDye ™ Terminator kit version 3.1 (PE Applied Biosystems, Warrington, England). Sequencing reactions were purified with Sephadex® G50 (GE-Healthcare-Pharmacia, Buckinghamshire, UK), and subjected to capillary electrophoresis on an ABI3130-XL (Thermo Fisher Scientific, Massachusetts, USA). DNA **s**equences were compared to those in GenBank (http://www.ncbi.nlm.nih.gov/BLAST). To identify the host species, the best match with identity of 98% or above was recorded.

2.4. Data analysis

Blood-feeding indices were calculated for female mosquitoes in each peridomestic habitat. The human blood index (HBI) was calculated for *Ny. darlingi* as the number of positive feeds on humans, divided by the total number of positive blood-fed mosquitoes (Garrett-Jones, 1964).

The forage ratio (w_i) (Hess et al., 1968) and selection index (B_i) (Manly et al., 1993) were calculated to verify the preference of mosquitoes collected for a particular host (Lardeux et al., 2007). The w_i for species *i* is the percentage of females that have engorged on a given host (o_i) divided by the percentage of the host in the total population available in the habitat (p_i): $w_i = \frac{o_i}{p_i}$. A value of ~1.0 indicates neither a preference nor avoidance, $w_i > 1.0$ indicates preference and $w_i < 1.0$ indicates avoidance.

The selection index (B_i) is a standardized forage ratio value: $B_{i=\frac{w_i}{\sum_{i=1}^{n} wi}}$, where w_i is the forage ratio for species *i* and *n* is the number of different blood sources available. Selection index values of ($1/n$) indicate no preference, values > 1 indicate preference and <1 indicate avoidance. The forage ratio (w_i) and the selection index (B_i) were

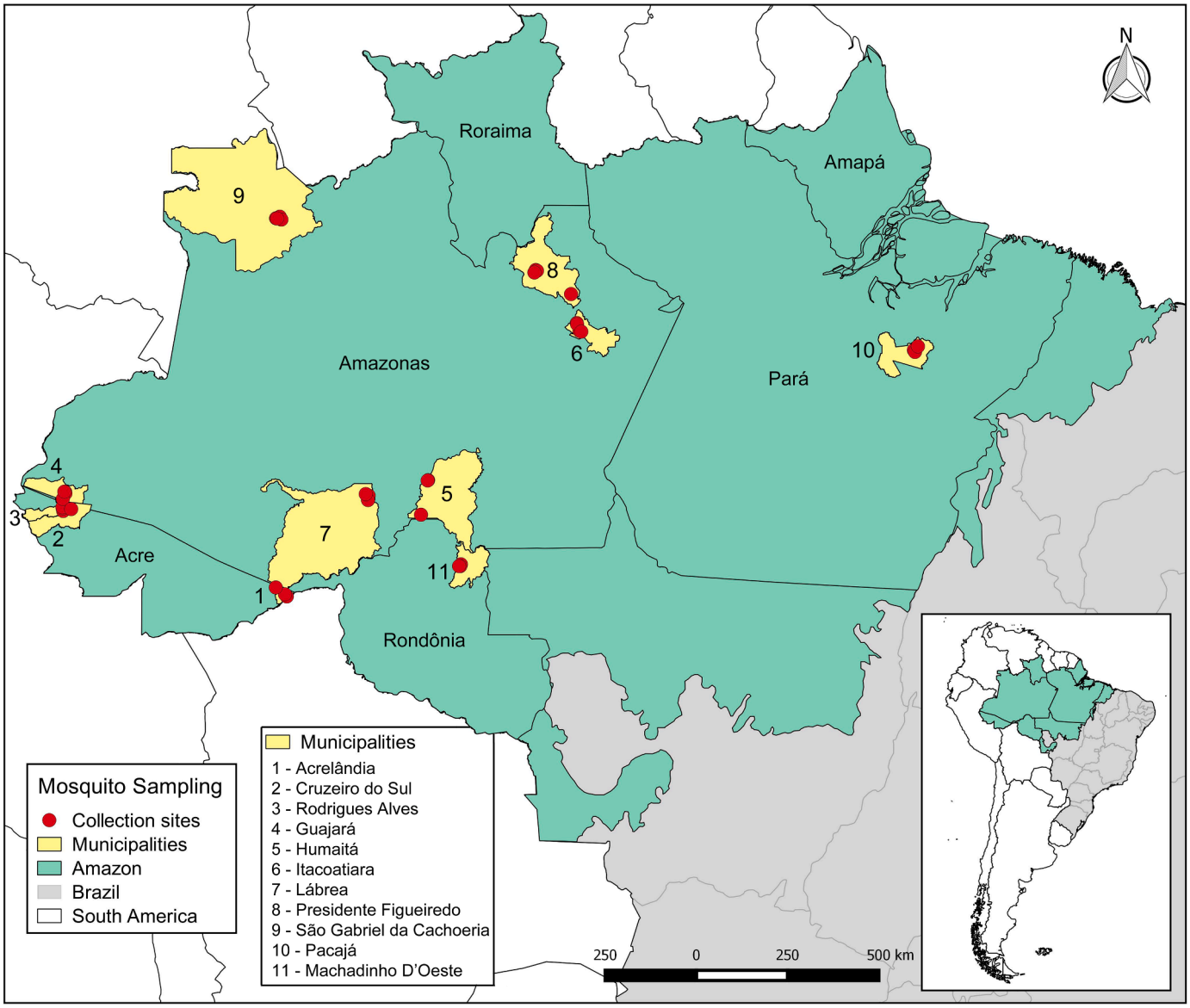

Fig. 2. Map of sampling locations across Brazilian Amazon states. The red dots inside the yellow areas represent the peridomestic habitats sampled in each municipality.

Table 2
Primers used to amplify fragments of the *Cytb, COI* and *16S* mitochondrial genes of vertebrates to identify the source of blood in the mosquito gut.

Primer	Sequence (5' to 3')	Length (bp)	Target	Ref.
Cytb (f)	GAGGMCAAATATCATTCTGAGG	≅ 450	Vertebrate	Townzen et al., 2008
Cytb (r)	TAGGGCVAGGACTCCTCCTAGT			
COI_short (f)	GCAGGAACAGGWTGAACCG	≅ 330	Vertebrate	Townzen et al., 2008
COI_long (r)	AAGAATCAGAATARGTGTTG			
16Smam1	CGGTTGGGGTGACCTCGGA	≅ 85	Mammal	Taylor, 1996
16Smam2	GCTGTTATCCCTAGGGTAACT			

computed only for blood sources identified in more than 10 females.

3. Results

With a sampling effort of 132 hours, 6,073 Anophelinae specimens were collected resting on the barrier screen; they consisted of 15 species. *Nyssorhynchus darlingi* dominated at 97.94%, followed by *Ny. braziliensis*, 1.47%, *Ny. albitarsis* s.l., 0.16%., and the remaining 12 species comprised 0.5%. Numbers of Anophelinae collected ranged from 10 in Acrelândia (AC-ACR), to 2,586 in Presidente Figueiredo (AM-PRE). Eleven species were each collected exclusively in a single municipality: *An.* near *fluminensis, An. minor, An. peryassui, Ny. albitarsis* s.l., *Ny. albitarsis* G, *Ny. albitarsis* s.s., *Ny. benarrochi* B, *Ny. deaneorum, Ny. nuneztovari* A, *Ny. oswaldoi* A and *Ny. rangeli* (Table 3). Few Culicinae specimens were collected, and they were excluded from analyses.

Apart from *Homo sapiens* (39.09%), the most common hosts in the surveys were *Gallus gallus* (chicken) (34.82%), *Bos taurus* (cow) (8.84%), and *Canis lupus familiaris* (dog) (8.19%); each of *Buballus buballis*

Table 3
Distribution and number of Anophelinae species collected using BSS in 34 peridomestic habitats within 27 rural communities in 11 municipalities, Amazon, Brazil, from 2015 to 2017.

Species	AC-ACR	AC-CRU	AM-GUA	AM-HUM	AM-ITA	AM-LAB	RO-MCH	PA-PAC	AM-PRE	AC-ROD	AM-SGC	%
Ny. albitarsis s.l.	–	10	–	–	–	–	–	–	–	–	–	0.16
Ny. albitarsis G	–	–	–	–	–	–	–	–	2	–	–	0.03
Ny. albitarsis s.s.	–	–	–	–	–	–	–	–	4	–	–	0.07
Ny. benarrochi B	–	–	–	–	–	–	–	4	–	–	–	0.07
Ny. braziliensis	–	–	–	55	–	33	–	–	–	1	–	1.47
Ny. darlingi	6	269	181	324	67	703	270	18	2577	275	1258	97.94
Ny. deaneorum	1	–	–	–	–	–	–	–	–	–	–	0.02
An. near *fluminensis*	–	–	–	–	–	–	–	–	–	–	1	0.02
Ny. goeldii	–	–	–	–	–	–	1	1	1	–	–	0.05
An. minor	–	–	–	–	1	–	–	–	–	–	–	0.02
*Ny.nuneztovari*A	–	–	–	–	–	–	–	–	2	–	–	0.03
Ny. oswaldoi A	–	–	–	–	–	–	1	–	–	–	–	0.02
An. peryassui	–	2	–	–	–	–	–	–	–	–	–	0.03
Ny. rangeli	3	–	–	–	–	–	–	–	–	–	–	0.05
Ny. triannulatus	–	–	–	–	–	–	–	1	–	1	–	0.03
Total	10	281	181	379	68	736	272	24	2586	277	1259	100

(buffalo), *Felis silvestris catus* (cat), *Sus scrofa* (pig), *Meleagris gallopavo* (turkey), *Equus caballus* (horse) and Anatidae represented less than 2% and were restricted to some habitats. Pet sylvatic animals, including macaws, parrots and monkeys, were relatively uncommon (~0.6%) (Fig. 3).

Primers targeting the vertebrate *Cytb* gene did not amplify one of the positive controls (horse), and occasionally amplified mosquito DNA. The *COI* primers amplified all controls and recovered the same hosts as *Cytb*; however, mosquito DNA was often amplified. To overcome the mis-targeting problem, a third set of primers were employed to amplify a small fragment of the *16S* region. The *16S* primers were used for mosquito samples that could not be identified by either *COI* or *Cytb* primers. The *16S* amplified vertebrate DNA only, including birds. All PCR positive samples were Sanger sequenced, and the vertebrate species was identified by BLAST comparison of the DNA sequence with those available in the GenBank data base.

A total of 6,073 anophelines was collected in BSS, of which 483 females (7.95%) were visually classified as blood-fed and 5,590 (92.05%) as unfed. For the blood source identification, all blood-fed, and a sample of unfed (2,795) specimens were PCR-tested (S1). Four-hundred-sixty-five visually classified as blood-fed females were identified successfully for the blood source (96.3%). Considering females which were visually classified as unfed but PCR-tested, 121 (5.23%) were found to be positive for vertebrate blood, whereas the blood source of 13 engorged females could not be amplified after several tests, using distinct PCR conditions and thermo-cycling profiles, and the Sanger sequence chromatograms of PCR amplicons from 5 females, returned DNA sequences with multiple peaks suggestive of mixed blood meals. No further analysis was conducted to distinguish among the multiple peaks. These 18 samples were excluded from the forage ratio and selection index calculations. Overall, we present blood meal analysis results for 586 female PCR-positive samples (483 original blood-fed, plus 121 classified as unfed but PCR positive, minus 18 excluded).

Results of DNA sequence comparison allowed accurate identification of 9 vertebrates: human (*Homo sapiens*), dog (*Canis lupus familiaris*), cow (*Bos taurus*), pig (*Sus scrofa*), buffalo (*Buballus bubalis*), horse (*Equus caballus*), chicken (*Gallus gallus*), turkey (*Meleagris gallopavo*) and a South American rodent, the green acouchi (*Myoprocta pratti*). Considering results of all peridomestic habitats together, 39.7% of females blood-fed on dog, followed by human (34%), cow (18.9%), pig (3.9%), buffalo (2.0%), horse (0.5%), chicken (0.5%), rodent (0.3%), and turkey (0.2%) (Table 4, Fig. 4).

Of the total of 586 female PCR-positive for vertebrate blood (Table 4), 564 (96.3%) were *Ny. darlingi*, 11 (0.9%), *Ny. braziliensis*, 3 (0.5%), *Ny. benarrochi* B, 3 (0.5%) *Ny. rangeli*, 2 (0.3%), *Ny. triannulatus* and, one of each of *An. peryassui, Ny. albitarsis* s.l., and *Ny. oswaldoi* A (0.2%). The other species were negative for blood using the *COI, Cytb* and *16S* primers.

The HBI values for *Ny. darlingi* (Table 5) ranged from 0.03 – 1.00, eliminating those habitats with fewer than 5 females tested. The HBI calculated for each peridomestic habitat, even in the same municipality, showed great variation.

Results of the forage ratio (w_i) and the host selection index (B_i) for *Ny. darlingi* are found in Table 6. Cow was the preferred host in 5 of 8

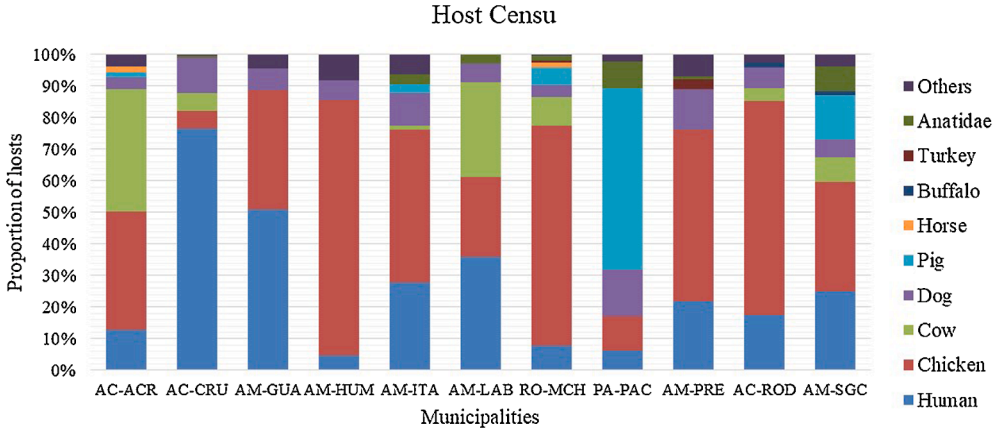

Fig. 3. Relative proportion of total number of vertebrates census in 34 peridomestic habitats within 27 rural communities in 11 municipalities, Amazon, Brazil, from 2015 to 2017.

Table 4
Anophelinae species and bloodmeal sources identified from PCR-tested females, collected in 34 peridomestic habitats within 27 rural communities in 11 municipalities, Amazon, Brazil, from 2015 to 2017.

Species	Buffalo	Chicken	Cow	Dog	Horse	Human	Pig	Rodent	Turkey	Total
Ny. albitarsis s.l.	–	–	–	–	1	–	–	–	–	1
Ny. albitarsis G	–	–	–	–	–	–	–	–	–	0
Ny. albitarsis s.s.	–	–	–	–	–	–	–	–	–	0
Ny. benarrochi B	–	–	–	3	–	–	–	–	–	3
Ny. braziliensis	–	–	1	2	–	4 [4]	–	–	–	11
Ny. darlingi	12	3	85 [21]	207 [20]	2	117 [72]	20 [3]	1	1	564
Ny. deaneorum	–	–	–	–	–	–	–	–	–	0
An. near *fluminensis*	–	–	–	–	–	–	–	–	–	0
Ny. goeldii	–	–	–	–	–	–	–	–	–	0
An. minor	–	–	–	–	–	–	–	–	–	0
Ny. nuneztovari A	–	–	–	–	–	–	–	–	–	0
Ny. oswaldoi A	–	–	–	–	–	1	–	–	–	1
An. peryassui	–	–	1	–	–	–	–	–	–	1
Ny. rangeli	–	–	3	–	–	–	–	–	–	3
Ny. triannulatus	–	–	–	–	–	[1]	–	1	–	2
Total	12	3	111	232	3	199	23	2	1	586

Number between brackets represents specimens considered unfed.

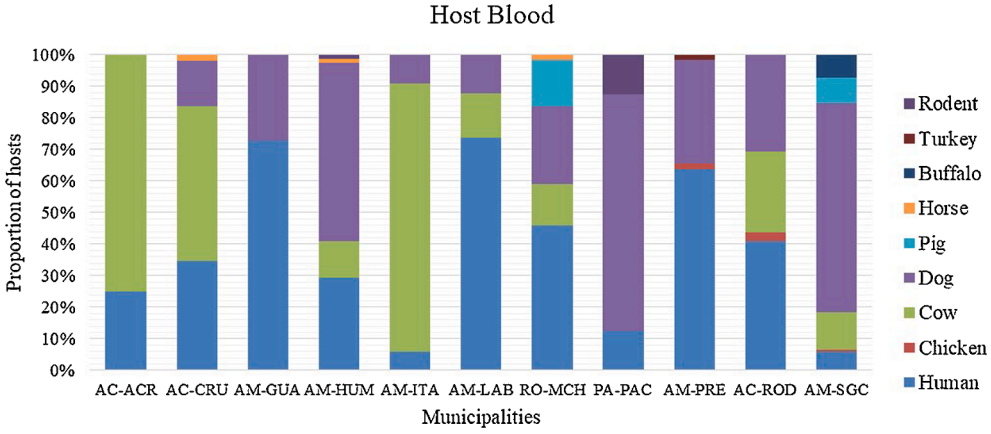

Fig. 4. Relative proportion of vertebrate blood identified in the mosquito abdomen of females collected outdoors in 34 peridomestic habitats within 27 rural communities in 11 municipalities, Amazon, Brazil, from 2015 to 2017.

Table 5
Human Blood Index (HBI) of *Nyssorhynchus darlingi* collected in collected in 34 peridomestic habitats within 27 rural communities in 11 municipalities, Amazon, Brazil, from 2015 to 2017.

Locality	Peridomestic habitat 1		2		3		4	
	N	HBI	N	HBI	N	HBI	N	HBI
AM-LAB	26	0.38	32	0.94	7	0.86	–	
AC-ACR	1	1.00	–		–		–	
RO-MCH	38	0.34	20	0.70	–		–	
PA-PAC	4	0.25	–		–		–	
AM-HUM	9	0.78	2	0.50	60	0.15	–	
AM-ITA	31	0.03	–		–		1	1.00
AC-ROD	40	0.38	–		–		–	
AC-CRU	8	0.38	10	0.50	29	0.31	–	
AM-GUA	2	0.50	7	0.86	13	0.69	–	
AM-PRE	10	0.90	29	0.69	14	0.29	7	0.57
AM-SGC	7	0.14	160	0.03	9	0.44	2	0.50

N= positive blood-fed mosquitoes
– = no human blood or no collection

peridomestic habitats where cows were present. In 13 of 22 peridomestic habitats, *Ny. darlingi* showed a preference (B_i) for dogs, and 7 of 25 habitats, for humans. Overall, dogs presented the highest number of blood-fed mosquitoes (Table 4), with the highest values (0.70, 0.86 and 0.96) found in AM-SGC (Table 6). Human was the second host in number of blood-fed mosquitoes (Table 4) with a range of B_i from 0.28–1.00 (Table 6). The preference for the blood source in each peridomestic habitat was usually a single animal, but in RO-MCH-1, *Ny. darlingi* shared a preference between humans and pigs, whereas in RO-MCH-2, this mosquito preferred both humans and dogs, among the hosts available. In AC-ROD-1, *Ny. darlingi* preferred dogs and cows, and in the AM-SGC-2, it prefers feeding on dogs and buffalos (Table 6; S2).

4. Discussion

Results of the analysis focused on host-seeking behavior of *Ny. darlingi* in peridomestic habitats of rural communities in the Brazilian Amazon, and confirmed that it is an opportunist species, blood-feeding on human, dog, cow, pig, buffalo, and other vertebrates. Similar results were found in studies conducted in other Latin American localities (Grieco et al., 2002; Zimmerman et al., 2006; Moreno et al., 2017). The values of the forage ratio and host selection index varied across localities investigated. Different findings across Latin America may be due to variation in the collection methods employed in different studies and host prevalence in each locality sampled. Despite differences, our findings in rural settlements across Brazilian Amazon, confirmed that *Ny. darlingi* exhibited multiple host affinity.

The BSS method was successfully employed to address the blood-feeding behavior of *Ny. darlingi* collected in peridomestic habitats in rural communities in the Brazilian Amazon and riverine communities in

Table 6
Forage ratio and host selection index of *Ny. darlingi* calculated based on blood source identifications and vertebrate census.

Ny. darlingi	Code	Forage ratio (*wi*) Peridomestic habitat				Selection index (*Bi*) Peridomestic habitat			
		1	2	3	4	1	2	3	4
Human	AM-LAB	0.87	2.19	3.43	–	0.08	0.63*	0.46	–
(*H. sapiens*)	AC-ACR	10.00	–	–	–	1.00	–	–	–
	RO-MCH	3.04	11.11	–	–	0.28*	0.53*	–	–
	PA-PAC	3.38	–	–		0.45	–	–	–
	AM-HUM	16.07	–	–	–	0.82*	–	–	–
	AM-ITA	0.08	–	–	6.50	0.00	–	–	1.00*
	AC-ROD	2.31	–	–	–	0.18	–	–	–
	AC-CRU	2.06	1.15	0.56	2.14	0.29	0.23	0.10	1.00
	AM-GUA	0.75	6.00	0.92	–	0.09	0.67*	0.33	–
	AM-PRE	3.36	3.86	1.40	–	1.00	0.64*	0.24	–
	AM-SGC	0.27	0.28	0.64	0.65	0.04	0.01	0.14	0.09
Dog	AM-LAB	1.92	1.31	4.00	–	0.18	0.38	0.54*	–
(*C. l. familiaris*)	RO-MCH	3.36	8.47	15.33	–	0.31	0.40*	1.00	–
	PA-PAC	4.05	–	–	–	0.55*	–	–	–
	AM-HUM	3.44	–	–	–	0.18	–	–	–
	AM-ITA	0.73	3.00	–	–	0.02	1.00*	–	–
	AC-ROD	4.80	–	–	–	0.37*	–	–	–
	AC-CRU	4.13	0.87	4.81	–	0.59*	0.17	0.90*	–
	AM-GUA	7.80	3.00	1.85	–	0.91*	0.33	0.67*	–
	AM-PRE	–	2.17	3.14	–	–	0.36	0.55*	–
	AM-SGC	6.67	15.94	3.86	–	0.96*	0.70*	0.86*	–
Cow	AM-LAB	7.67	–	–	–	0.73*	–	–	–
(*B. taurus*)	RO-MCH	0.88	–	–	–	0.08	–	–	–
	AM-ITA	30.71	–	–	–	0.97*	–	–	–
	AC-ROD	6	–	–	–	0.46*	–	–	–
	AC-CRU	0.83	2.99	–	–	0.12	0.60*	–	–
	AM-SGC	–	1.32	–	6.50	–	0.06	–	0.91*
Pig	RO-MCH	2.62	1.41	–	–	0.24*	0.07	–	–
(*S. scrofa*)	AM-SGC	–	0.49	–	–	–	0.02	–	–
Buffalo	AM-SGC	–	4.60	–	–	–	0.20*	–	–
(B. *buballis*)									

* Indicate the preferred host
– No blood-fed mosquitoes

peri-Iquitos, Peru, where the forage ratio and selection index indicated high preference for Galliformes over humans (Moreno et al., 2017). Curiously, in Amazonian Brazil, chickens were also abundant, much more so than dogs, in 9 of 11 municipalities (Fig. 3; present study); yet only 3/564, or 0.54% of *Ny. darlingi* collected and tested, fed on chickens (Table 4). Dogs were similarly abundant (roughly 5% of total vertebrates) in both study areas, thus local availability of hosts is a poor explanatory factor. These findings might suggest local adaptation of a preference for chicken blood meals in Amazonian Peru in contrast to dog blood meals in Amazonian Brazil. Interestingly, an early study in Amazonian Brazil by Deane et al. (1949), collecting *Ny. darlingi* indoors, found similar human and dog preferences to the outdoor mosquito collection results in the present study.

For blood-feeding PCR identification, the specimens were kept dry in silica gel for several months. To overcome potential problems, we employed primer pairs designed to amplify short DNA region of the *Cytb, COI* mtDNA and *16S*. In this way, 9 vertebrate host species were identified. In addition, this study showed a lower rate of blood meal identification comparing to other studies, however, most of the studies with high identification rates used only visually engorged mosquitoes whereas we tested a substantial number of unfed mosquitoes. If analyses of this study were restricted to the mosquitoes visually considered engorged, information of 5.23% (n = 121) would be lost, since sequencing can identify traces of blood hosts. Furthermore, these additional samples provide a more thorough evaluation of the HBI and the proportion of various host blood meal sources.

The HBI of *Ny. darlingi* presented a high range of values, from 0.03–1.0, similar to the range found by Zimmerman et al. (2006). According to Lardeux et al. (2007), as HBI shows variation between locations, it is more accurate to refer to the HBI of a population *versus* that of a species. The presence of multiple hosts in a blood meal when the PCR product is sequenced by the Sanger method usually increases the cost and time of the experiment as the product must be cloned. These challenges have limited studies to single blood meal detection only, making it more challenging to illuminate overall feeding patterns. This study detected about 0.17 % of specimens containing multiple blood meals. This low value does not necessarily reflect the actual numbers of patent blood meals taken and it cannot distinguish multiple cryptic meals. Despite the low value, this feeding behavior is important because it can increase the frequency of human contact or even reduce when the blood meals are taken from different vertebrate sources that are not considered *Plasmodium* reservoirs.

Many authors have found that the time of night anophelines feed on blood varies depending on several factors, such as species density, seasonality, host availability, among others (Barros et al., 2007; Zimmerman et al., 2013; Barbosa et al., 2016). Tadei and collaborators (1998) found that *Ny. darlingi* blood fed all night but peaked at dusk and at dawn, whereas in a separate study, this species showed a wide range of temporal blood feeding patterns in Amapá, Brazil (Voorham, 2002).

A recent investigation compared the host affinity of *Anopheles gambiae* Giles (anthropophilic) and *Anopheles arabiensis* Patton (zoophilic) and revealed that differences observed were caused by the collection habitats, i.e., indoors *versus* outdoors collections (Orsborne et al., 2018). One possible limitation of our study was that we only collected outdoors, near houses where people were dwelling. Thus, our results of the complete vertebrate host preference of *Ny. darlingi* can be biased by the populations examined. Consequently, our findings may represent blood-feeding behavior of an exophilic population of *Ny. darlingi*. An important question that can be raised by our findings is related to the impact of LLIN for malaria control. It is well known that the impact of

this intervention is most effective against endophilic *Ny. darlingi*, and it does not provide any protection against exophilic *Ny. darlingi* (Prussing et al., 2018; Saavedra et al., 2019). However, our study revealed that females collected outdoors fed mainly on human, dog and cow with *Ny. darlingi* as the dominant species in the peridomestic habitats of Amazonian rural settlements. Recently, Sallum et al. (2019) employing mathematical modelling calculations, demonstrated that exophagic *Ny. darlingi* has a low vector competence for *P. vivax*, contrasting with a moderate to high vectorial capacity. Despite these contrasting characteristics, the level of malaria transmission was high in several localities included in the study (MCH, Rondônia state and SGC, Amazonas state), demonstrating that the involvement of exophagic *Ny. darlingi* in *Plasmodium* propagation cannot be neglected. The preference for human blood detected in our study is important to understand the moderate to high vectorial capacity of *Ny. darlingi* populations in the above-mentioned study. Furthermore, it is important to evaluate how the availability of alternative blood meals can impact mosquito survival and the malaria risk potential in endemic areas.

Anopheline female collections were conducted across a range of outdoor locations across the Brazilian Amazon. The opportunistic tendency of *Ny. darlingi* to select the host from which it will secure a blood meal can be influenced by, local host availability biomass, and ecological factors, but little is known about their respective contributions. In addition, intrinsic genetic factors might also explain the host affinity of the species. However, it will be necessary to conduct additional investigations to demonstrate the influence of intrinsic factors on the behavioral plasticity and anthropophagy of *Ny. darlingi*. Such knowledge will be critical for delineating the effectiveness of control programs and the impact on malaria control strategies.

5. Conclusion

This study has demonstrated that *Ny. darlingi* exhibited an appetite for multiple hosts, depending upon location and available hosts. The forage ratio (w_i) and host selection index (B_i) revealed that the host preference can vary, and that dogs and cows are attractive alternate hosts to humans in peridomestic habitat in Amazonian Brazil. Despite the sequencing method employed in the study that can underestimate the number of multiple blood meals, the high percentage of successful PCR amplifications demonstrated that field-collected specimens dried in silica gel provided usable host blood DNA for effective analysis.

Supporting Information

S1. Dataset. PCR specimens. The file shows all specimens that were PCR tested. For each mosquito analyzed, information of locality, species, blood source. (XLSX)

S2. Dataset. Selection index. The file shows all specimens of *Ny. darlingi* that have forage ratio, selection index and threshold calculated for each peridomestic site. (XLSX)

Funding

This work was supported by Fundação de Amparo à Pesquisa do Estado de São Paulo, FAPESP [grant # 2014/26229-7 to MAMS, EM, ESB], [grant # 2014/09774-1 to GZL], [grant # 2016/08562-6 to SSN]; National Institutes of Health, USA [grant # R01 AI110112 to JEC]; and Conselho Nacional de Pesquisa, CNPq [grant # 301877/2016-5 to MAMS].

CRediT authorship contribution statement

Sandra Sayuri Nagaki: Conceptualization, Investigation, Formal analysis, Visualization, Writing - original draft. **Leonardo S.M. Chaves:** Formal analysis, Resources, Writing - review & editing. **Rossana Verónica Mendoza López:** Formal analysis. **Eduardo S. Bergo:** Resources. **Gabriel Z. Laporta:** Resources, Writing - review & editing. **Jan E. Conn:** Conceptualization, Funding acquisition, Writing - review & editing. **Maria Anice Mureb Sallum:** Project administration, Conceptualization, Methodology, Resources, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Acknowledgements

We are indebted to Tatiane Porangaba de Oliveira for her suggestions for establishing the PCR protocols employed for blood source identification and assistance, and to Brisa Sallum de Paula for her friendly assistance in sorting samples in the laboratory. Eduardo Evangelista, Leonardo S. Chaves and Liliane Milanelo provided DNA samples for PCR positive controls.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.actatropica.2020.105751.

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