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Larval habitats of *Anopheles* species in a rural settlement on the malaria frontier of southwest Amazon, Brazil

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ABSTRACT

Rural settlements are social arrangements expanding in the Amazon region, which generate disturbances in the natural environment, thus affecting the ecology of the species of *Anopheles* and thus the malaria transmission. Larval habitats are important sources for maintenance of mosquito vector populations, and holding back a natural watercourse is a usual process in the establishment of rural settlements, since the formation of micro-dams represents a water resource for the new settlers. Identifying characteristics of the larval habitats that may be associated with both the presence and abundance of *Anopheles* vectors species in an environment under ecological transition is background for planning vector control strategies in rural areas in the Amazon. *Anopheles* larvae collections were performed in two major types of habitats: natural and flow-limited water collections that were constructed by holding back the original watercourse. A total of 3123 *Anopheles* spp. larvae were captured in three field-sampling collections. The majority of the larvae identified were taken from flow-limited water collections belonged to species of the *Nyssorhynchus* subgenus (92%), whereas in the natural larval habitats a fewer number of individuals belonged to the *Stethomyia* (5%) and *Anopheles* (3%) subgenera. The total of *Nyssorhynchus* identified (1818), 501 specimens belonged to *An. darlingi*, 750 to *An. triannulatus* and 567 for others remaining species. In addition, 1152 could not be identified to subgenus/species level, because they were either in the first-instar or damaged. The primary vector in areas of the Amazon river basin, *An. darlingi*, was found exclusively in man-made habitats. Statistical analysis display *An. triannulatus* with specialist behavior for characteristics of man-made habitats. Modifications in the natural environment facilitate the rise of larval habitats for species with epidemiological importance for malaria in the region. This study showed that man-made habitats flow-limited water collections from dry lands could be a factor associated with the increase of *An. darlingi* and *An. triannulatus* populations, and other *Nyssorhynchus* species as well in endemic areas of the Amazon Region.

1. Introduction

Human malaria constitutes an important challenge for Public Health authorities, as it is actively transmitted in 97 tropical and subtropical countries, with more than 210 million cases being registered in the world in 2015 (WHO, 2015). The disease is caused by protozoan species of the genus *Plasmodium*, which are transmitted by 41 dominant vector species of the genus *Anopheles*; nine species are endemic from the Neotropical Region (Sinka et al., 2012). As regards the importance of the *Anopheles* species in the dynamic of transmission, it is noteworthy that some are considered primary vectors and others, secondary or auxiliary vectors. This division is determined by the vector capacity of the species, which depends on environmental and population factors such as: the density of the female population of the vector in relation to the human population; the daily probability of a human host being bitten by females of the mosquito vector; the duration of the period of extrinsic incubation of the pathogen; the proportion of females with sporozoites in their salivary glands. The parameters associated with the fitness of the mosquito vector, for example, the survival and density of the females, are determined by environmental factors which act fundamentally during the development of the larvae (Brady et al., 2016; Moller-Jacobs et al., 2014). The presence of larval habitats is

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a ubiquitous factor for the occurrence and maintenance of the density of the vector population, being directly involved in the rate of the spread of malaria. The main neotropical vector is the mosquito *Anopheles* (*Nyssorhynchus*) *darlingi*, which is of widespread distribution in South America, including the Amazon Region (Hiwat and Bretas, 2011). There are, however, other endemic species, which can act as primary and secondary vectors in the continent (Forattini, 2002; Sinka et al., 2010).

As regards malaria in South America, in 2013 eight countries accounted for more than 90% of all notified cases. Of that total, Brazil contributed 42%; Venezuela 18%; Columbia 12%; Peru 10%; Guyana 8%, and Bolivia 2% (OPAS, 2014). In Brazil, it is to be observed that 99% of the cases occurred in the states which together compose the *Amazonia Legal* (MS, 2014), a region in which the pattern of the geographical distribution of the cases and of the respective zones of risk for the disease is heterogeneous and accompanies the patterns of the occupation of the soil and the use of the land, excepting as regards the occurrence of malaria among indigenous populations (Oliveira-Ferreira et al., 2010). The Amazon Basin is a source of energy and mineral resources which has, historically, attracted a demographic flux of a social and economic character (Tauil and Daniel-Ribeiro, 1998). The patterns of the transmission of malaria thus depend both on the type and on the proportion of the degradation of the area of native forest and, consequently, on the predominant economic activities. In this way, the dynamics of the transmission of malaria in the zones of extractive economic activities (rubber extraction, prospecting, timber exploitation), farming zones, urban centers (and suburban perimeter) and indigenous reserves are distinct, because they involve human populations with specific demographic, social and economic characteristics, and determine different ecological factors and respective communities of *Anopheles* (Fearnside, 2005; Lounibos and Conn, 2000).

Environmental changes in the rural settlements have been employed to define the dynamics of the transmission of malaria in agricultural frontier areas (Castro et al., 2006). In these areas, the process of the occupation of the soil for the development of agriculture and cattle-raising favors the contact of the human population with the *Anopheles* mosquitoes, leading to outbreaks, epidemics and endemic situations, in accordance with the environmental and social-demographic factors associated with economic activities in the rural settlement (Barbosa et al., 2014; Silva-Nunes et al., 2012, 2008; Silva-Nunez et al., 2010). These alterations generally lead to an increase in the density of the species that possess a higher degree of synanthropy and can be involved in the transmission of *Plasmodium* as well (Barros et al., 2011a,b; Moutinho et al., 2011; Conn et al., 2002; Lourenço-de-Oliveira et al., 1989). In these environments which are undergoing intense processes of modification, the habitats of the larvae are profoundly affected by intervention of original watercourses. *Anopheles darlingi* is one of the species favored by the human presence and the respective environmental changes, which lead to the rise of larval habitats capable of maintaining dense populations of the vector (Hiwat and Bretas, 2011), in particular the construction of lakes for fish farming (Reis et al., 2015a, 2015b; Maheu-Giroux et al., 2010; Vittor et al., 2009; Costa et al., 2010). In the published literature records, *An. darlingi* is frequently described as a species that has some degree of dependence on the presence of forest, or fragments forest (Barros et al., 2011a,b; Barros, 2007; Moutinho et al., 2011; Nagm et al., 2007; Vittor et al., 2006).

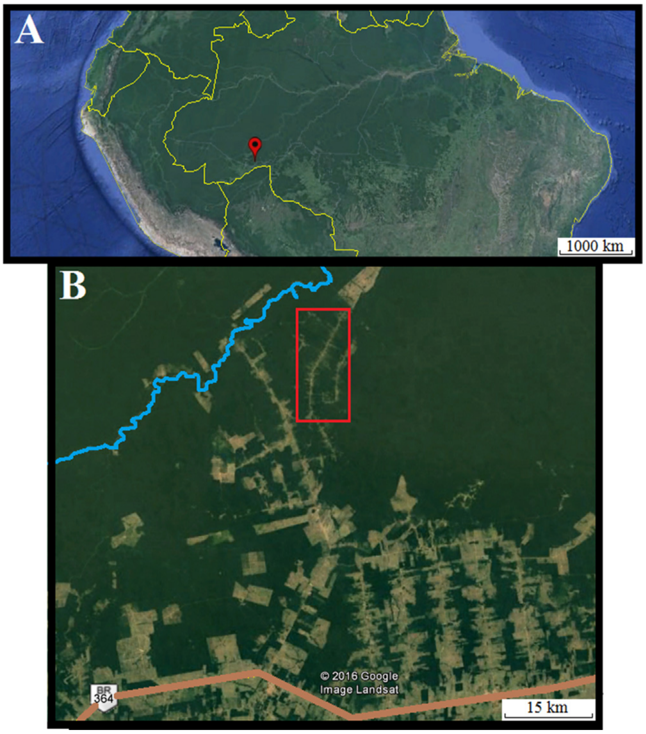

Fig. 1. Study area. A: overview of the Amazon River Basin and its tributaries in South America, indicating the location of Remansinho settlement in Brazil. B: Remansinho settlement (red rectangle), especially the river Iquiri (in blue) and the federal highway BR 364 (in brown). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Source: ©2016 Google-Images ©2014 TerraMetrics, Nasa, Cartographical data ©2016 Maplink.

The structure of the *Anopheles* communities in aquatic environments depends on a multiplicity of such factors as: shade, water flow, temporality (by rainfall seasonality), associated vegetation, trophic structure of the habitat and physical-chemical and biochemical conditions of the water (Barros et al., 2011a; Reis et al., 2015a; Sánchez-Ribas et al., 2015; McKeon et al., 2013; Vittor et al., 2009, 2006; Nagm et al., 2007; Tadei et al., 1998). Other factors affect the dynamic of the larval *Anopheles* communities, such as the volatile substances which emanate from the aquatic habitats, a recognized oviposition stimulant for pregnant females (Rejmánková et al., 2005), and the availability of hosts, and the distance between the larval habitat and the human dwellings, which represent a source of blood for anthropophilic species (Smith et al., 2014). For the purpose of determining the characteristics of the aquatic habits and the presence of *Anopheles* mosquitoes, this present study investigated larval habitats, represented by original and man-made collections of land water, in rural settlements in Brazilian Amazon. As representing natural habitats, *igarapés*, located inside the forest edge, were selected. As representing man-made habitats, ponds for water reservoirs, constructed by means of the deviation and damming-up of the watercourse of the *igarapés*, in open areas. Some particular characteristics of the landscape were selected and some physical-chemical properties of the water measured. In the light of the data published in the literature on investigation into mosquito larvae of epidemiological importance, the hypothesis tested was: the communities (sampled) of *Anopheles* larvae present in natural habitats differ from those of man-made habitats.

2. Materials and methods

2.1. Study area

The current Remansinho rural settlement is located in the municipality of Lábrea, southern Amazon state, Brazil, near the border of Bolivia (Fig. 1). Initially a rubber tapper community, became a rural settlements composed of a major farming community and four smaller communities. The major settlement is situated along a 60 km-long unpaved road originating from the BR-364 interstate highway, while the other four are situated along minor secondary unpaved roads. The colonization of major Remansinho community started in 2007, with the human houses having either a complete or incomplete wooden walls and thatched roofs, a few houses are made of bricks with a zinc metal roof. There is no electricity or piped water supply in the area by the time of the study (Barbosa et al., 2014).

The Remansinho settlement is divided into small agricultural plots situated along a 40-km unpaved road, which is linked to the federal highway BR-364. The distribution of the plots obeys the so-called 'fish-bone" layout (Castro et al., 2006). The settlement is located in a region of dryland forest, which is approximately 10 km distant from the Iquiri River, the main tributary of the river basin (Fig. 1). During the study period (2012–2013), it was not observed extensive agricultural activity, only small crop and livestock subsistence farming. The presence of domestic and wild animals was constant in the area. Other economic activities practiced in the settlement were the extraction of wood, coconut *açaí* and Brazilian nuts.

Similar to other areas south Amazon River, the climate is equatorial, humid, with annual average temperature of 26.4 °C, and intense rainfall from November to March with annual average of 2318 mm. In accordance with the data of the Center for Weather Forecasting and Climate Studies (CPTEC, 2014), for the three-year period 2011–2013, the rainfall and temperature regimes were typical of the equatorial-tropical region with the first semester presenting higher rainfall, with a monthly average above 200 mm; the second semester being the dry season with averages below 50 mm for the months of June, August and September, and the increase in rainfall occurring as from the month of November with an average above 200 mm (Appendix A). Deforestation indicators obtained from Deforestation Monitoring Project in the Amazon Satellite (PRODES) indicated fluctuations in annual rates of deforestation over the past 15 years throughout the Amazon region (Appendix B). In the state of Amazonas, the municipality of Lábrea was the one who had the highest rates in the period from 2002 to 2014, followed by other municipalities also situated in south of the state: Boca do Acre, Apuí and Manicoré (PRODES, 2016).

The population census and the epidemiological profile of malaria in Remansinho settlement was conducted by Barbosa et al. (2014) between the years 2010 and 2013, recording an average of 260 inhabitants (min: 165, max: 290). During the study there was a decrease in the prevalence of *P. vivax*, however 50% of infections were subclinical. The groups with the highest risk for malaria were identified as the new settlers coming from areas without active malaria transmission.

2.2. Study design

A cross-sectional study was undertaken, which included a preliminary analysis in April 2012 followed by three fieldworks. Field collections for the present study were carried out in the major community named Ramal Remansinho. For the study, we examined larval-habitats located in areas along the major road of the settlement. Twelve localities were analyzed in preliminary analysis by environment modification, positivity for *Anopheles* sp., breeding-site dimension, and logistic for fieldwork, according to the hypothesis of the study. Eight localities were selected: four in areas that have undergone less intense deforestation and four were in a modified environment. Landscape 1, 2, 3 and 4 represented natural watercourses; 5, 6, 7 and 8 were man-made ponds by diverting and holding back a natural watercourse (Fig. 2). Landscape was treated as a proxy variable for larval habitats. Inherent in the study design, the choice of the original larval habitats demanded more difficulty, especially the lack of reference to a nearby residence, such as with man-made ponds. Field collections were conducted in May 2012, April 2013 and May 2013. Other details regarding the collections of water and of the distances between the breeding-sites and the dwellings are presented in Appendix C.

2.3. Stratification of the sampling units

The original and man-made habitats were stratified in independent sampling subunits for the collection of larvae and the measurement of the environmental variables. The division into subunits rendered from 20 to 30 sites of capture, according to the length and configuration of the habitat. The distance between the sampling points was approximately two meters: a 2 m-distance was considered sufficient to neighboring subunits were less influenced by physical and light disturbance caused by the previous collection, in an attempt to maintain the independence of the points of the same habitat. Only the first subunit of each habitat was georeferenced in UTM, using the World Geodetic System 84 (WGS84). A total of 595 subunits of the natural and man-constructed habitats were sampled. The distribution of the number of sampling subunits by habitat and period is presented in Appendix D.

2.4. Larval collection

The captures of the larvae were standardized in accordance with the recommendation of the Health Surveillance Secretariat of the Ministry of Health (SVS-MS, 2011). Aluminum recipients of 10-cm diameter and 350 ml volume, provided with a 1.5 m long handle, were used. In each subunit, for the collection of water (original and man-made), nine dips were made (Appendix E). The larval collections in each habitat were undertaken from 8:30 am to 11:30 am and from 2:30 pm to 5:30 pm, totaling 42 h collection effort during eight consecutive days in each fieldwork. The collections were alternate between an original habitats in one day followed by collections in a man-constructed habitat in the consecutive day, in an attempt to decrease climatic influences for comparison purposes. The principal author (PRM), with the help of a local field technician, is responsible for all collections and measurements of physical-chemical characteristics of the water. The larvae were captured with pipettes and transferred to plastic flasks identified by collection site and respective subunits, conserved in 80% ethanol and transported to the Public Health Entomology Laboratory (Laboratório de Entomologia de Saúde Pública). The specimens were identified morphologically as to larval stage, subgenus and, whenever possible, species level, using the identification keys proposed by Forattini (2002).

2.5. Measurements of the sampling subunits

The sampling subunits were characterized in terms of type of vegetation covering (primary forest; pasture; absent), water flow (presence; absence), turbidity (transparent; murky, using a Sechi disk) and types of refuges for the immature forms. The refuges for the immature forms present in each habitat were divided into four categories, according to the arrangement of the associated vegetation in water level: bank (has no associated vegetation); floating (aquatic vegetation with roots fixed on the surface of the water); submerged (vegetation with its roots above the level of the water, with its upper parts covered by the water level); emerging (vegetation with its roots fixed in the substrate below the water level, with its upper part above the water-level). A schematic representation of these refuge types is illustrated in Appendix F. The variable vegetation cover was used as an indirect measure of the shade provided. In the original watercourses, the captures were undertaken against the water flow.

The physical-chemical variables, such as the pH, temperature (°C) and conductivity (μS/cm), were measured using a digital

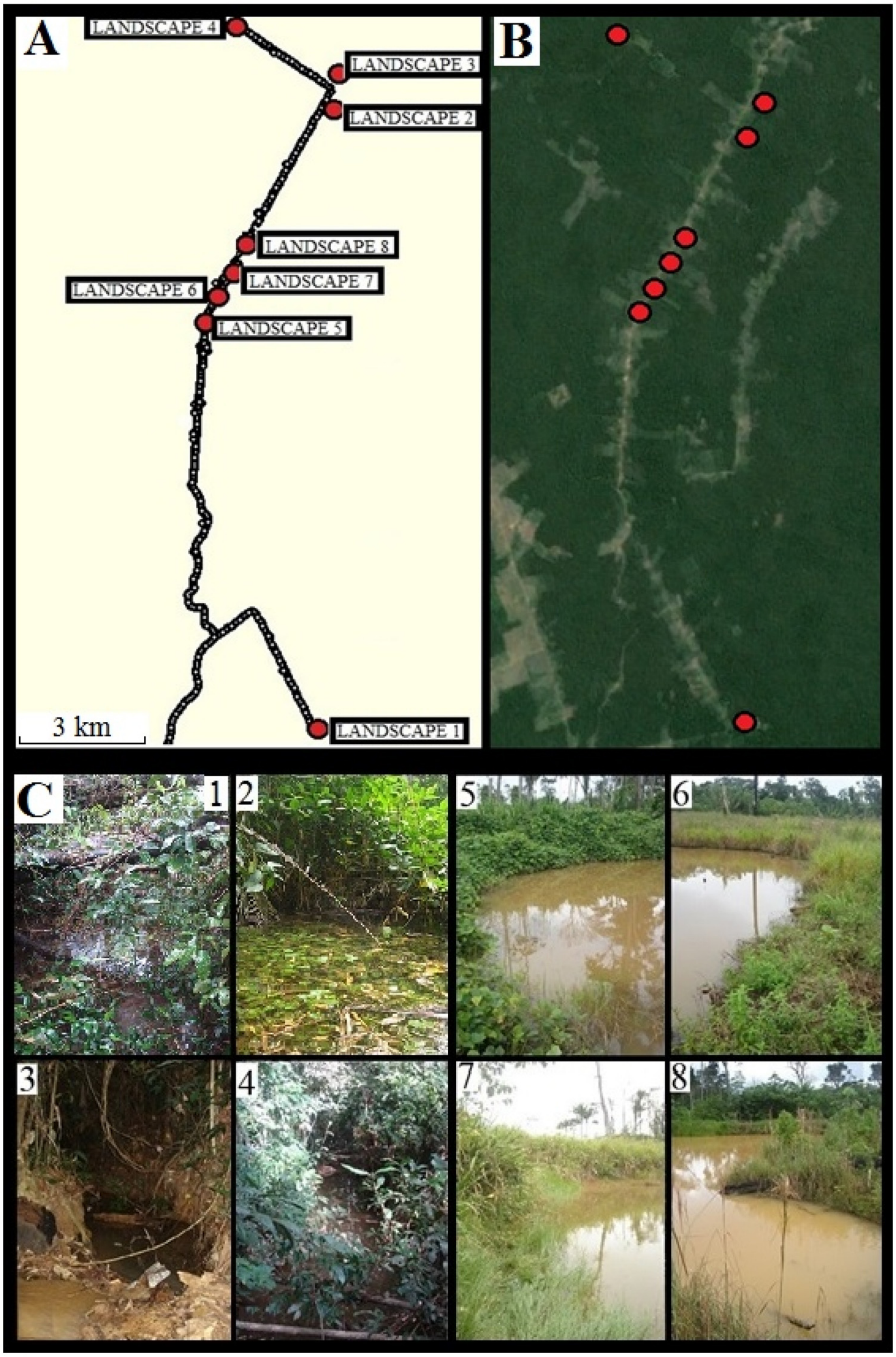

Fig. 2. A: diagram representing a branch of the rural settlement and the landscapes/breeding-sites selected for the study; B: a satellite image and the landscapes (source: ©2014 Google-Images). C: photographs of representatives of the habitats sampled in the rural settlement: 1–4 original breeding-sites; 5–8 man-made breeding-sites.

pocket multi parameter meter with one electrode, ITMMA-35-Instrutemp model. To measure the quantity of dissolved oxygen (mg/L), a digital meter was used, OD-4000-Instrutemp model. The instruments were calibrated and standardized in accordance with the manufacturers' recommendations, and the measurements were taken between 12.00 and 14.00 h for each fieldwork-day, minimizing random errors for comparison purposes.

2.6. Statistical analyses

The statistical analyses were undertaken using the Stata 11 program (Data analysis and statistical software – StataCorp LP, College Station, TX). The values of the outliers of the variables were observed by means of *boxplot* graphs. This phase of the analysis was undertaken separately for each collection of water, by period. It was decided to use the average of the water collection, attributing

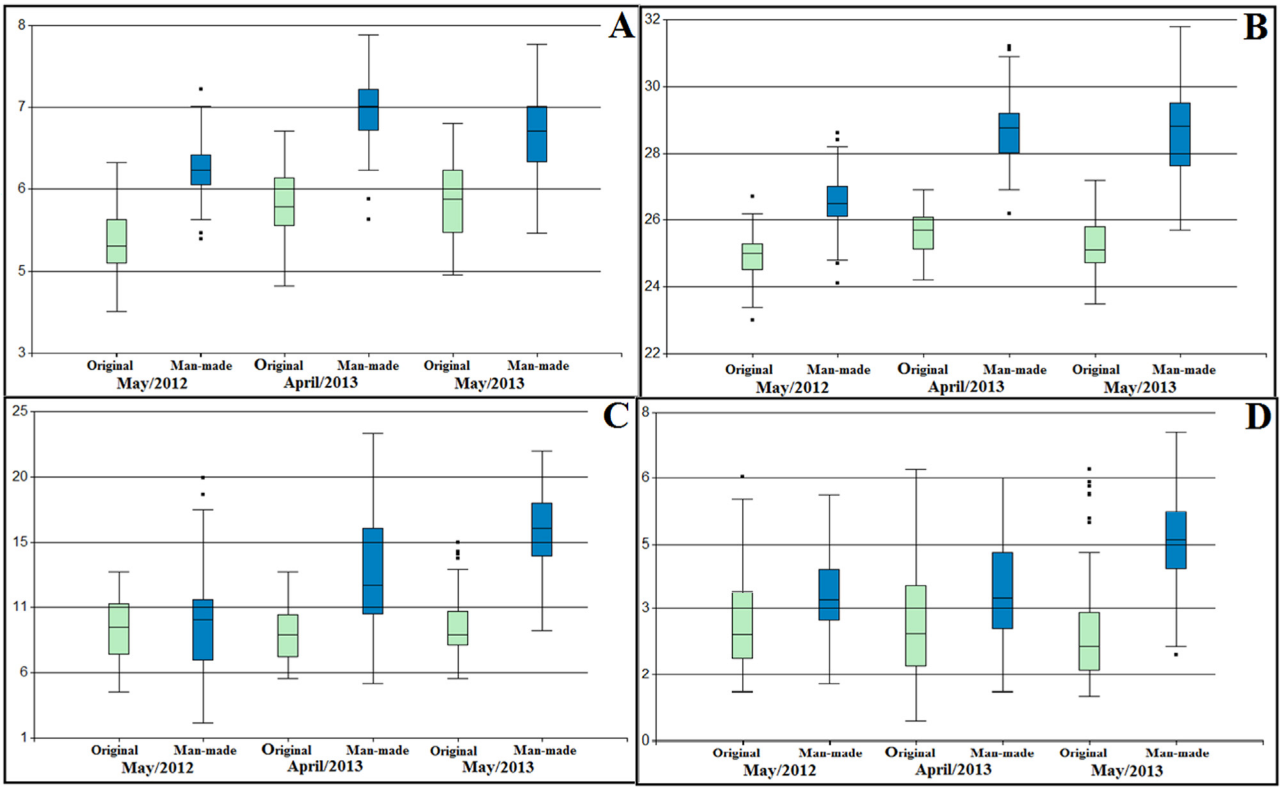

Fig. 3. *Boxplot* graph for the physical-chemical variables of the water: (A) pH; (B) temperature in °C; (C) conductivity in µS/cm; (D) dissolved oxygen in mg/L, by type of breeding-place and month and year of collection. The comparisons of the variables by Kruskall Wallis' test reveal p values of less than 0.001 except for dissolved oxygen, April 2013 (p = 0.002) and conductivity in May 2012 (p = 0.510).

to it values which were outstanding individually, due to probable systematic errors (Engels and Diehr, 2003).

The captures were made in the same habitats for reasons of logistics and knowledge of the field, though due to the seasonal variation the subunits were not the same throughout the collection-period. It was presumed that the three periods collections were independent of each other, as the exact spatial position of the subunits in each habitat was not maintained throughout the three fieldworks: exogenous factors such as the alteration in the water-level due to the rainfall regime and of different degrees of anthropic stress lead to a highly dynamic situation, inherent to biological properties, a condition which was not monitored by the methodology used here (Hurlbert, 1984). No analyses for the assessment of seasonal patterns of the habitats were undertaken, due to the insufficient number of fieldworks.

The Kruskall-Wallis test was used to compare the physical-chemical variables (as they presented non-normal distribution) of the collections of water, both original and man-made. Pearson's chi-squared test was used for the analyses of the landscape variables, by means of contingency tables. Multicolinearity between the physical-chemical variables was verified by Pearson's linear correlation coefficient and by Principal Component Analysis (PCA).

In addition to checking the hypothesis of the differences between the types of investigated larval habitats, also it was performed analysis to verify associations between the landscape variables and the physical-chemical characteristics of the water with taxonomic groups collected. For univariate analyses, we used coefficient of linear correlation for the physical-chemical, and contingency tables for categorical variables, checking presence/absence of the respective groups identified larvae. Also histogram was performed for larval species. For multivariate analysis, in view of the fact that the histograms were asymmetrical, i.e. were skewed to the right (right-skewed histogram) due to the high number of zeros (subunits with the absence of larvae), overdispersion was observed, a common characteristic in ecology studies (Martin et al., 2005). Negative Binomial regression appears to be appropriate for these assumptions and analysis of vectors invertebrate organisms (Reis et al., 2015a; Kaizer, 2015; Sriwicha et al., 2015; Imbahale et al., 2012). Robust regression was selected, according to Stata 11 command. Two different standards were used for the insertion of the independent variables in Negative Binomial regression, thus establishing the best fit. In view of the fact that not all the possible biological parameters related to the larval ecology were measured, an initial analyses was constructed which brought together all the independent variables obtained during the collections, then a reduced model was opted, for which the independent variables were selected by univariate Negative Binomial analysis (Zar, 2010). A cut-off value for p of less than 0.2 was decided on (that is to say, a maximum of 20% of statistical association as a criterion for selection by the univariate Negative Binomial). Beyond the preceding method for the usual Negative Binomial regression, an approach was applied which took the large number of null values into consideration by Negative Binomial zero-inflated. Zero-inflated model permits corrections of estimates of the probability of occurrence for false-negative (failure to record a species when in fact it is present) (Tyre et al., 2003). It was thus possible to assess the adequacy and the goodness-of-fit for the models. Also was conducted a control for the sampling effort, considering the time interval between the three field visits, by two approaches: a three months data, aggregating the three fieldworks (n = 295); and two months data, aggregating April and May 2013 (n = 200). According to the results, only man-made habitats subunits were considered for the association analyses.

Table 1
Distribution of the variables: vegetation cover, water flow, turbidity and refuge, for the type of breeding-place and month of collection.

Variables	May/2012		April/2013		May/2013	
	Original	Man-made	Original	Man-made	Original	Man-made
Vegetation cover[*]						
primary forest	100	–	100	–	97	–
pasture	–	73	–	97	3	89
absent	–	22	–	3	–	11
Waterflow[*]						
present	59	–	62	6	67	3
absent	41	95	38	94	33	97
Turbidity*						
transparent	91	–	84	–	88	–
turbid	9	95	16	100	12	100
Refuge[*]						
bank	65	25	74	10	72	18
floating	19	–	10	–	12	–
submerged	16	17	16	26	16	32
emerging	–	53	–	64	–	50

[*] Values of p of less than 0.001 for the tests of association by Pearson's χ-squared test.

3. Results

3.1. Characteristics of the water collections

The landscape pattern of the original and man-made habitats is given in Table 1. The environments with the lowest degree of disturbance present primary forest coverage, while the man-made ones were in environments whose forest covering had been removed, and consequently presented pasture growth. The water was standing and relative turbid in those man-constructed, flow-limited water collection habitats. On the other hand, in the natural habitats, the water was transparent and slow moving with the presence of subunits where the water flowed freely. As regards refuges for the larvae, a significant absence of vegetation was observed on the banks of the least disturbed breeding-places: banks with a total absence of vegetation predominated, but with the presence of floating and submerged refuges, the latter resulting of the growth of bordering vegetation, which came in contact with the water surface. In the man-constructed habitats, the most common larval refuge was represented by emerging refuges: pasture post-deforestation in contact with the water level, which increased by damming-up of the watercourse.

Fig. 3 shows the boxplot graphs of the physical-chemical variables, with evident differences between the average values obtained for the original and man-constructed habitats in the three fieldworks. The only exception being the conductivity of the water in May 2012 when no statistically significant difference was found between the values measured. The natural habitats presented more acid waters, at a lower temperature, lower conductivity and a lesser amount of dissolved oxygen at the water surface. Other statistical information regarding the collections of water is presented in Appendix G.

3.2. Distribution of *Anopheles* species

A total of 3123 *Anopheles* spp. specimens were obtained during the three field collections. Of that number, 1818 individuals belonged to the subgenus *Nyssorhynchus*, distributed as follows: 501 were of *An.* (*Nys.*) *darlingi*, 750 of *An.* (*Nys.*) *triannulatus* and 567 were identified to subgenus level as *An.* (*Nyssorhynchus*) spp. The latter group was categorized as "remaining species of *Nyssorhynchus*". Specimens belonging to other subgenera of *Anopheles* were captured: 52 specimens of the subgenus *Anopheles*, and 101 of the subgenus *Stethomyia*. Another 1152 specimens were identified to the genus *Anopheles* level, because they were either first-instar larvae (n = 1118) or were severely damaged (n = 34).

Fig. 4 shows the distribution of subgenera and species by sampling unit (larval habitat) for each fieldwork. Species belonging to the subgenus *Nyssorhynchus* were found in man-constructed, flow-limited habitats. *An. triannulatus* was collected more frequently in units 5 and 6, whereas *An. darlingi* predominated in 7 and 8, except during the April 2013 collection. In the larval habitats classified as natural habitats, a lower amount of *Anopheles* specimens were collected, however, they belonged to *Stethomyia* and *Anopheles* subgenera, with presence of *Nyssorhynchus* only in the Landscape 1, May/2012. Distribution of the first-instar specimens is in Appendix H (they were captured in great numbers in the man-constructed habitats).

3.3. Association analyses

Multivariate analyses were undertaken for the larvae gathered in three taxonomic groups, namely: *An. darlingi*, *An. triannulatus*, and remaining species of *Anopheles* (*Nyssorhynchus*) spp. Histograms for taxonomic groups are presented in Appendix I.

Analyses of multicolinearity between the physical-chemical variables for three months data (n = 295) presented only a statistically significant relationship (>25%) between pH and temperature, with a value for linear correlation test of 0.41; and only one principal component with a significant eigenvalue (1.57), accounting for approximately 40% of the variance between pH and temperature (0.553 and 0.647, respectively). For two month data (n = 200), presented also only a significant linear correlation between temperature and conductivity (approximately 0.30); and two principal component: a first (*eigenvalue* = 1.45), accounting for approximately 36% of the variance between pH and temperature (0.498 and 0.505, respectively), and a second (*eigenvalue* = 1.11), accounting for approximately 27% of the variance between temperature and dissolved oxygen (0.545 and 0.687, respectively).Values of coefficient of linear correlation are presented in Appendix J.

The four physical-chemical variables were used for the multivariate models. For the Negative Binomial usual, refuge was considered a dummy independent variable, as it presented larvae in more than one response class. For the Negative Binomial zero-inflated, refuge was considered control variable for count the excess of zeros, by biologically explain the absence of larvae in subunits exposed to sunlight. Table 2 gives the results of the Negative Binomial and Negative Binomial zero-inflated, by the two sample size approaches (n = 200 and n = 295). Considering the adjustments for the insertion of the independent variables, it can be noted that no significant alterations were observed, suggesting that the both models used added no information which might explain the vari-

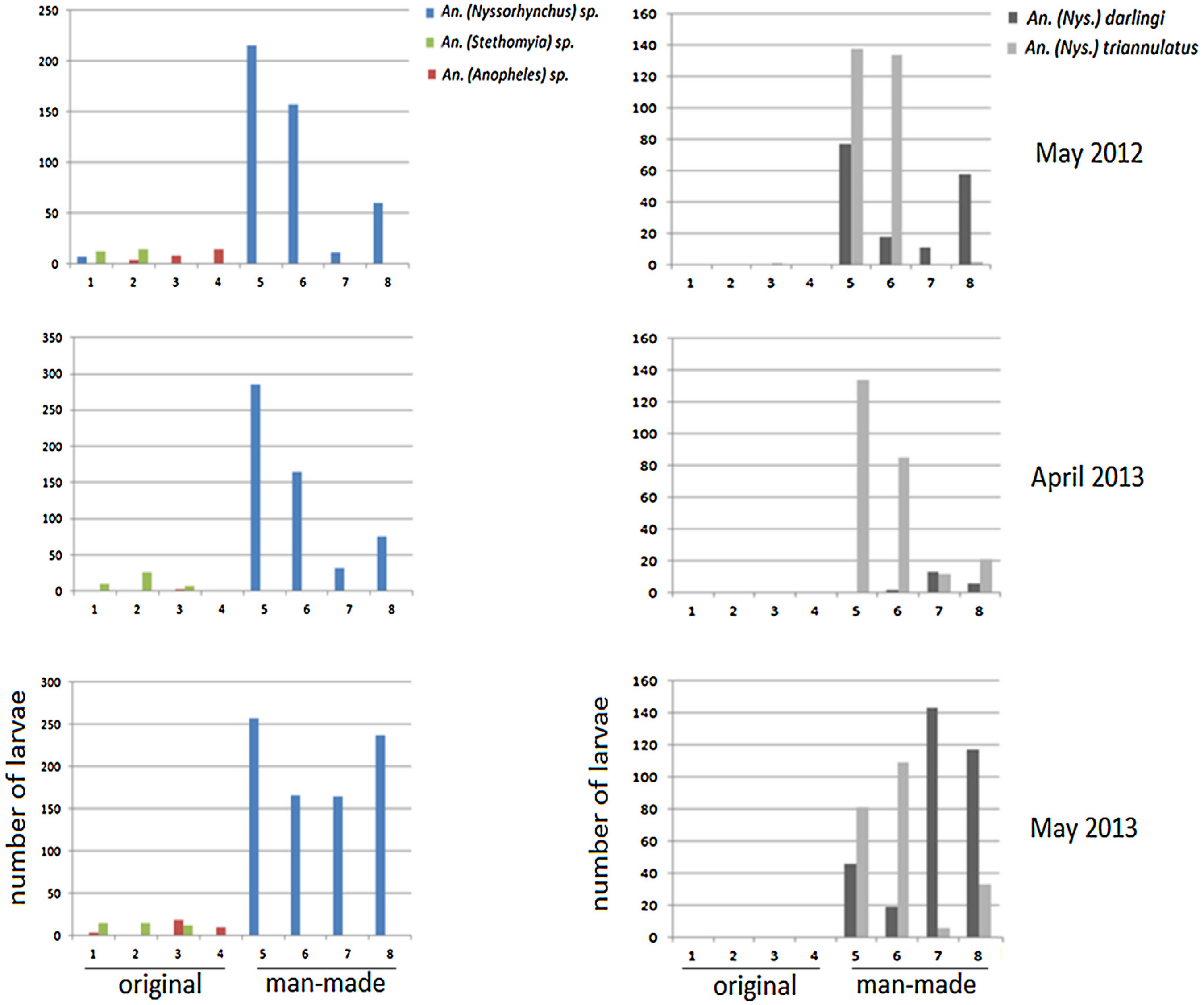

Fig. 4. Distribution of immatures of *Anopheles* sp. by major kind of habitat and month/year of collection. On the left side the distribution data is relative to the subgenus. On the right side, the distribution data is for *An. darlingi* and *An. triannulatus*.

ability of the independent variables (Appendix K), so only results for cut-off model are presented. The values of incidence rate ratio (IRR) and respective effect size were used as a measure of association, but not for the magnitude of the same.

The variable refuge was the one which best explained the presence and counts of larvae from the man-made habitats. For the four physical-chemical variables used, the statistical pattern of *An. triannulatus* was stable for the number of the variables associations ($p < 0.05$), both Negative Binomial models (except for pH) and sample size approaches. *An. darlingi* presents heterogeneity for the number of associations, if controlled by sample size approach: for the two months data ($n = 200$), the group presents statistical significance ($p < 0.05$) for three physical-chemical variables (pH, temperature and conductibility); for the three months data ($n = 295$), the number of significant associations was lower (only pH for Binomial Negative usual and only temperature for BN zero-inflated). The remaining species of *Nyssorhynchus* group presented a similar statistical pattern with *An. darlingi* group, however with greater number of significant associations for the three months data ($n = 295$).

4. Discussion

The Amazonian Forest is the habitat for various *Anopheles* species, including those associated with the transmission of malaria (Pimenta et al., 2015). The selective pressure, represented by the alterations of primary forest environment, facilitates both the invasion and dispersion of mosquito communities with differing degrees of synanthropy, including species that are vector of *Plasmodium* (Barata et al., 2012; Barros et al., 2010; Galardo et al., 2007; Quiñones et al., 2006). In the areas of pristine forest of Brazilian Amazon *Anopheles* species – including the principal neotropical vector *An. darlingi* – are rare or even absent compared with others Culicidae groups (Hutchings et al., 2016, 2013, 2005). This happens because the larval habitats are deeply shaded, rich in organic matter resulting from leaf litter, and with presence of water flow, all factors that hinder the development of the immature stages and respective maintenance of population of vectors (Gil et al., 2007). With the process of deforestation, increase of the incidence of sunlight on the ground and damming up of watercourses, the characteristics of the collections of water and the communities structure of

Table 2
Statistical Association for the three taxonomic groups of *Nyssorhynchus* for the type of Negative Binomial Regression (usual and inflated zero) and sample size approaches (n = 200 e n = 295).

Groups	Independent Variable	Two months data (n = 200)				Three months data (n = 295)			
		Negative Binomial	p	Negative Binomial Zero Inflated	p	Negative Binomial	p	Negative Binomial Zero Inflated	p
An. darlingi	Refuge (submerged)	51.30	0.000	na	na	19,34	0,000	na	na
	Refuge (emerging)	83.01	0.000	na	na	31.89	0,000	na	na
	pH	0.53	0.008	0.59	0.020	0.62	0.026	–	0.070
	Temperature (°C)	1.50	0.000	1.55	0.000	–	0.062	1.13	0.040
	Conductivity (μS/cm)	1.08	0.049	1.13	0.001	–	0.774	–	0.132
	Dissolvedoxygen (mg/L)	–	0.143	–	0.227	–	0.072	–	0.533
An. triannulatus	Refuge (submerged)	26.64	0.001	na	na	18.07	0.000	na	na
	Refuge (emerging)	40.05	0.000	na	na	30.19	0.000	na	na
	pH	–	0.117	1.49	0.050	–	0.141	1.43	0.026
	Temperature (°C)	0.79	0.004	0.76	0.001	0.73	0.000	0.70	0.000
	Conductivity(μS/cm)	1.04	0.020	1.04	0.031	1.08	0.000	1.07	0.001
	Dissolvedoxygen (mg/L)	–	0.325	–	0.278	–	0.481	–	0.736
Anopheles (*Nyssorhynchus*) spp.	Refuge (submerged)	30.01	0.000	na	na	50.90	0.000	na	na
	Refuge (emerging)	47.73	0.000	na	na	83.75	0.000	na	na
	pH	–	0.516	–	0.198	2.01	0.000	2.22	0.000
	Temperature (C)	–	0.152	0.84	0.025	1.37	0.000	1.29	0.000
	Conductivity (μS/cm)	–	0.076	–	0.283	1.15	0.000	1.15	0.000
	Dissolvedoxygen (mg/L)	0.82	0.006	0.84	0.003	0.84	0.006	0.86	0.018

Negative Binomial: Incidence Rate Ratio (IRR). *An. darlingi*: Prob > chi2 = 0.0215; for *An. triannulatus* and the third group: Prob > chi2 = 0.0000. All the models presented a p value < 0.0000 for the variable refuge inflated for the excess of zeros. na = not applicable: refuge was considered control variable for zero-inflated model.

aquatic organisms can change. Taking into account the increasing availability of human hosts near an adequate water collection to support new aquatic communities, the area can be invaded and occupied by opportunistic mosquito species, thus infesting an area where previously found in low densities. This ecological succession is widely documented for the *Nyssorhynchus* subgenus, particularly *An. darling* (Gil et al., 2015, 2007; Fernández et al., 2014; Turell et al., 2008; Tadei and Dutary-Thatcher, 2000; Tadei et al., 1998, 1988, 1983; Lourenço-de-Oliveira et al., 1989). According to Hiwat and Bretas (2011), the immature stages of *An. darlingi* is favored by some degree of deforestation, and develop in aquatic habitats subject to partial shading, with temperature and pH varying, respectively, between 20 and 28 °C, and 6.5 and 7. Remarkably, the beginning of the process of changes in the ecological and community structures of the environment of the tropical Amazon forest favors the increase in larval habitats and density of *Anopheles* vector species, in the same way the maintenance of the original mosquito biodiversity in areas of ecological transition could be a factor of protection to avoid outbreaks of the disease (Hahn et al., 2014a; Laporta et al., 2013). Instead, the environmental disorder of Brazilian Amazonia advances due to the efforts of the present-day lobby for the reduction of the area of this biome in favor of farming and cattle-raising activities (Castelo, 2015). It is noted that the context for malaria in the Amazon, as well as others emerging infectious diseases, is a circumstance of a social-economic-political picture, composed by different motivations (Confalonieri et al., 2014; Hahn et al., 2014b).

Changes in the original courses of rivers and streams make water resources available for the settlements even in periods of drought, and the consequent damming up of water resources leads to the creation of ponds, which can become surrounded by new growth vegetation that are tolerant to the post-deforestation conditions. The new trophic structure dependent on the available resources and the respective physiological conditions of the aquatic habitats (Rejmánková et al., 2006), and this dynamic directly influences the communities of holometabolous insects with aquatic larval development, including *Anopheles* mosquitoes and the *An. darlingi*. For this species, Barros and Honório (2015), from studies performed in dry-land agricultural settlements in the Amazon, make a point of the high density in larval habitats arising from pasture and secondary growth, surround by forest fragments, these areas being associated with the spatial distribution of malaria; otherwise, completely deforested sites were absent for larvae. The authors hypothesize that the shade provided by the remaining forests are a key factor for the choice of habitats for oviposition. Transitional ponds, situated on the forest edge, harbored the highest prevalence of *An. darlingi* larvae, whereas the other types of larval habitats presented a smaller number of specimens or even none at all. On the other hand, Vittor et al. (2009) showed that the impact of deforestation on *Anopheles* aquatic communities in Peruvian Amazon was associated with the presence of *An. darlingi* larvae in areas with less than a quarter of remaining forest vegetation: the species was not found in areas with a larger percentage of the original forest cover. The presence of water plants, the size of the breeding-site and the proportion of secondary vegetation were determinant for the presence of *An. darlingi*. The authors hypothesize that factors associated with algal community and possible emanated compounds can act as attractive for pregnant females. The two research groups establish correlation between the beginning of deforestation and the increase in population of *An. darlingi*, but differ on the issue of the dependence of the species regarding primary forest. In this present study, the larval habitats that were situated inside the forest were negative for *An. darlingi*. However, by damming up of a natural watercourse with the construction of small weirs for the creation of ponds for family use, associated with the presence of secondary vegetation, offered appropriate conditions for the development of the immature stages of *Nyssorhynchus* species in general and *An. darlingi* in particular. This study does not establish a methodology to verify relation with remaining forest vegetation and density of larvae in man-made habitats. Furthermore, the absence of *An. darlingi* in those larval habitats located inside the forest can be caused by the size of the larval sampling in the natural habitats. The small sample size can have been affected by the limitations of the collection method, because it was restricted to the margins of the water bodies, and required the use of a long handle attached to a recipient that allowed the collector to reach the water. In addition, the larval collections inside the forest were restricted to a few streams, which may also explain the absence of *An. darlingi* from the natural habitats investigated (Conn and Ribolla, 2015).

The distance between larval habitat and dwellings determines the site for laying eggs (Reis et al., 2015a; Smith et al., 2014; Barros et al., 2010; Barros, 2007). In Remansinho settlement, the residents constructed artificial ponds in extra-domiciliary environment nearby their houses, because they use them as a water source for the dry season. The fact that the distance between the house and the pond is short, the contact between *Anopheles* mosquitoes and humans can increase. A mosquito community can encompasses several species, which can be non-vector or vector as well. *Anopheles* species can be infected by *Plasmodium*, however only a limited number of them can actively participate in the dynamic of the transmission of the agent depending on their vector capacity (Cohuet et al., 2010; Lounibos and Conn, 2000).

Anopheles triannulatus was found in greatest number in flow-limited larval habitats of the settlement. This species is frequently found in flow-limited larval habitat (Conde et al., 2015; Barros et al., 2010), and seems to play an important role in the transmission of malaria in transitional areas of the Amazon forest (Gil et al., 2015; Rios-Velásquez et al., 2013; Galardo et al., 2007). The presence of various *Anopheles* species competent for *Plasmodium* infection in a particular region can increase the complexity of the transmission dynamics, because several species may be acting as vectors in distinct spatial and temporal setting (Barros et al., 2012). Thus the presence of both *An. darlingi* and *An. triannulatus* in habitats nearby the houses seems to indicate that malaria transmission could occur in both peridomiciliary and extra-domiciliary environment in Remansinho: to the settlement population, the behavior to remain in residence and to practice activities related to forest had similar risk for malaria (Barbosa et al., 2014).

The presence of *An. darlingi* and *An. triannulatus* in the habitats sampled for this study was associated with turbid water and low bordering vegetation in extra-domiciliary environment. The flow-limited habitat with standing water was an important determinant for the presence of these two species (Barros et al., 2011b). This variable is important for the presence of the microorganisms associated with the habitat and is the basis for the "Flush-Out" theory, which proposes that the intensity of the rainfall and the consequent increase in the water flow in the breeding-sites are responsible for the larval mortality and the resulting reduction in the adult population (Barros et al., 2011a). The flush-out problem is not observed in artificial ponds, because during the rainy season there occurs a rise in water-level and the habitat is thus brought into contact with the undergrowth vegetation resulting from the degradation of the forest, thus creating an emerging refuge for the larvae (Appendix F). As regards the variable classified as refuge, it may be observed that this was an important determining factor for the presence of *Anopheles* species (Reis et al., 2015a; Grutters et al., 2015). Whereas the presence of larvae of the species of *Nyssorhynchus* was associated with the emerging and submerged vegetation found in the degraded habitats, specimens of the *Stethomyia* and *Anopheles* subgenera occurred in forest streams, with fresh, slow-moving water, deeply shaded, with floating plant debris and deep bank. *Anopheles* (Anopheles) *forattini* occurs in forest areas in the Amazon River basin. The larval habitat occupied by the species is streams, rich in floating plant debris, with slow-moving water, inside inundated forest (Hutchings and Sallum, 2001). Other *Anopheles* (*Stethomyia*) species that occur in the Amazon likely occupy similar larval habitats, for instance, the non-identified *Anopheles* subgenera specimens.

Results of the multivariate regression analysis demonstrated importance of the presence of refuge for taxonomic groups analyzed. For physical-chemical variables, *An. trianulatus* (group with the largest number of individuals collected), presented no distinction according to the Binomial Negative model and sample size approach, with similar values of associations, suggesting more specialist behavior for this species. The increasing of temperature suggests a limiting factor for larval counting on subunits, while the decrease in water acidity can provide conditions for their development (the latter variable only observed in the zero-inflated models). Contrariwise, *An. darlingi* did not show the same results when considering the two sample design approaches: for the two months data (n = 200), the change of physical-chemical indicators of water (according to environmental disturbance) favors the species at higher temperatures and low values of pH water. For three month data (n = 295) the results suggest a generalist behavior for physical-chemical conditions. For the group defined as *Nyssorhynchus*, identification of the totality of the larvae at species level might present more sensitive results, seeing that this group might include organisms of various species for the man-made habitats investigated. Some limitations can affect the associations of multivariate regression analysis according to the methodology to collected information in the field, such zero values and random errors in measurements of variables. However, considering that the same effort was used for the species sampled, for the results presented in this study the *An. triannulatus* group presents more specialist behavior than *An. darlingi* group for the physical-chemical conditions of man-made habitats (McKeon et al., 2013). *Anopheles darlingi* seems to be a more tolerant species for the physical-chemical conditions of a man-constructed habitat. This is an important characteristic for species associated with zones of ecological transition (Barros and Honório, 2015; Barros et al., 2011a,b; Vittor et al., 2009). In addition, results of the present study can help to understand why *An. darlingi* is the primary vector in degraded forest areas of the Amazon River basin. In some areas the natural environment has been intensively modified by human activities and those modifications seems to drive the selection of subpopulations with particular genetic and behavioral characteristics (Alimi et al., 2015; Conn and Ribolla, 2015; Angêlla et al., 2014).

Finally, results of the present study showed that both the degradation of natural forest and the new environment arrangements produced effects in the displacement of populations of the species autochthonous to the region with establishment and dispersion of species more tolerant to the new ecological condition. The knowledge of the ecological conditions that determine either the presence or the absence of *Anopheles* species competent to transmit *Plasmodium* is an important contribution to the planning of vector control in areas of rural settlement in the Brazilian Amazon. The identification of potential larval habitats is a challenge and, when standardized, larval control should be integrated into the other interventions such as the control of adults and the use of impregnated mosquito nets, thus establishing a basis for the control and elimination of malaria in Amazon River basin (Conn and Ribolla, 2015).

Declarations

None

Conflict of interests

The authors declare that they have no conflicting interests.

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Appendix A.

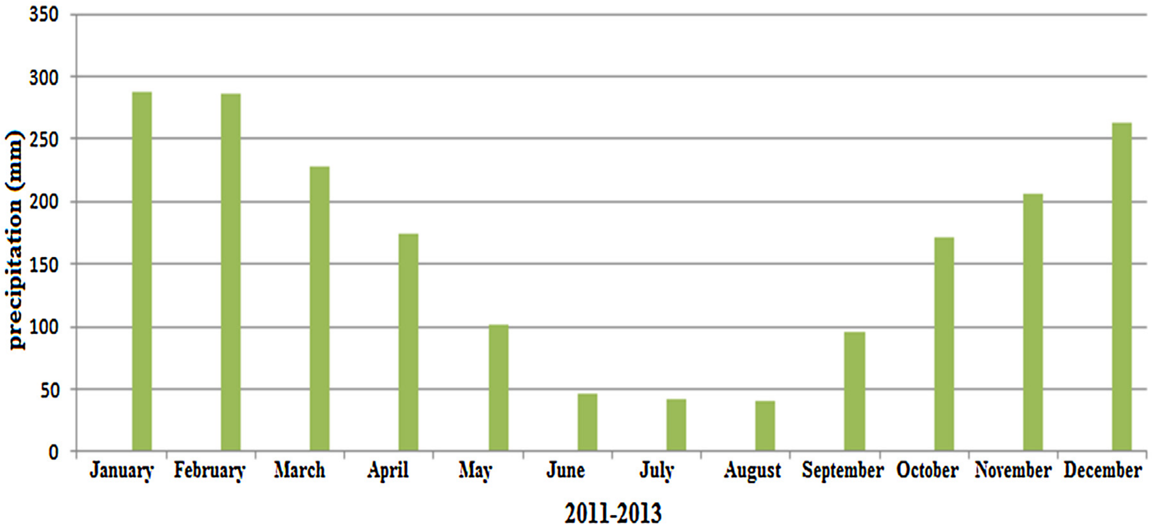

Fig. A1. Monthly-average precipitation (mm) for the three years (2011, 2012 and 2013).

Source: CPTEC, 2014.

Appendix B.

Table B1
Relative variation between annual deforestation rates, for the Brazilian states that constitute the Amazon, 2000–2015.

Year\State	Acre	Amazonas	Amapá	Maranhão	Mato Grosso	Pará	Rondônia	Roraima	Tocantins	Amazônia Legal
2000–2001	−23%	4%		−10%	21%	−21%	8%	36%	−23%	0%
2001–2002	111%	40%	−100%	13%	2%	43%	16%	−76%	12%	19%
2002–2003	22%	76%		−8%	32%	−5%	16%	420%	−26%	17%
2003–2004	−32%	−21%	84%	−24%	14%	24%	7%	−29%	1%	9%
2004–2005	−19%	−37%	−28%	22%	−40%	−33%	−16%	−57%	72%	−32%
2005–2006	−33%	2%	−9%	−27%	−39%	−4%	−37%	74%	−54%	−25%
2006–2007	−54%	−23%	30%	−6%	−38%	−2%	−21%	34%	−49%	−18%
2007–2008	38%	−1%	156%	101%	22%	1%	−29%	86%	70%	11%
2008–2009	−34%	−33%	−30%	−35%	−68%	−24%	−58%	−79%	−43%	−42%
2009–2010	55%	47%	−24%	−14%	−17%	−12%	−10%	112%	−20%	−6%
2010–2011	8%	−16%	25%	−44%	29%	−20%	99%	−45%	−18%	−8%
2011–2012	9%	4%	−59%	−32%	−32%	−42%	−11%	−12%	30%	−29%
2012–2013	−28%	11%	−15%	50%	50%	35%	21%	37%	42%	29%
2013–2014	40%	−14%	35%	−36%	−6%	−20%	−27%	29%	−32%	−15%
2014–2015	−10%	54%	−58%	−16%	40%	0%	41%	−32%	6%	16%

Source: PRODES, 2016.

Appendix C.

Table C1
Characteristics of the eight water collections (breeding-sites investigated), Remansinho settlement, Lábrea, Amazonas, Brazil.

Larval Habitat	Water Collection Configuration	Approximate area (m^2)	Approximate distance from dwelling (m)	Geo-referencing
1	meanders	50–40	80	9°29′34.40”S66°32′24.21”O
2	meanders	70–60	150	9°21′23.22”S66°32′11.86”O
3	meanders	40–30	100	9°21′06.07”S66°31′55.41”O
4	meanders	40–30	1500	9°20′24.42”S66°33′18.29”O
5	ponds	80–70	10	9°23′59.75”S66°33′55.31”O
6	ponds	180–150	10	9°23′15.50”S66°33′20.09”O
7	ponds	60–50	20	9°23′03.57”S66°33′14.05”O
8	ponds	170–150	10	9°22′46.62”S66°33′09.05”O

Configuration: meander: natural curse (*igarapé*); pond: course obstructed by human factor, forming a microdam. **Aproximatte area**: for natural breeding-site: distance between the banks, multiplied by the length (this length corresponds only to subunit spaces of observation, and not the total size of the respective watercourse); for artificial breeding-site: conisdering that the dimension may assume a circumference, due to the expansion of the water surface, was estimated diameter, and applied to the circle area formula ($\pi.r^2$). Approximate values for April and May 2013; **Approximate distance from dwelling (m)**: the distance between the residence of the landscape and the nearest point of breeding-sites (the first subunit). Approximated values for May 2012. Both measures (Area and Distance), as well **Geo-referencing**, were obtained using a GPS GARMIN.

Appendix D.

Table D1
Distribution of the number of sampling subunits according to the classification adopted for the breeding places and month of collection, Remansinho settlement, Lábrea, Amazonas, Brazil.

Type of Habitat	Subunit by habitat			Total by habitat
	May/2012	April/2013	May/2013	
Natural				
1	20	20	20	
2	30	30	30	
3	20	20	20	
4	30	30	30	
Subtotal	100	100	100	300
Man-made				
5	20	20	20	
6	30	30	30	
7	15	20	20	
8	30	30	30	
Subtotal	95	100	100	295
Total	195	200	200	595

Appendix E.

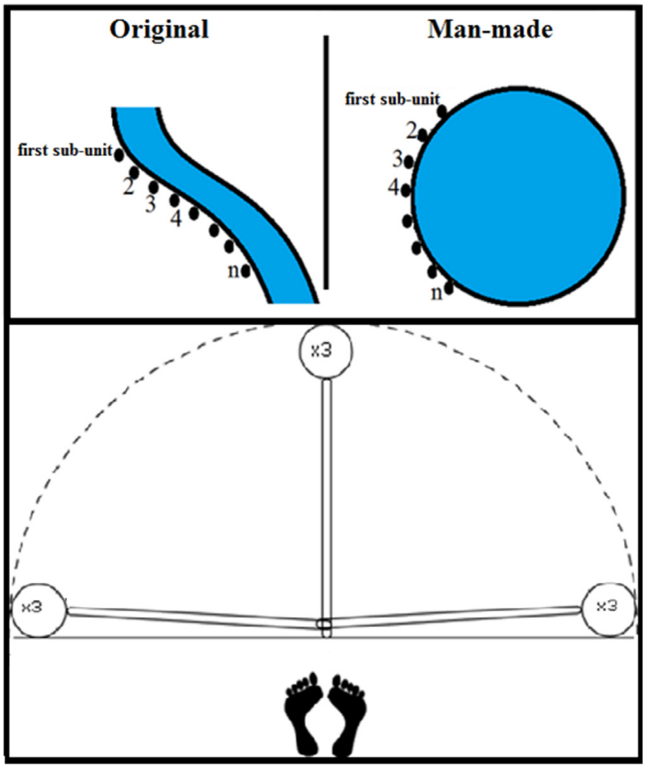

Fig. E1. Upper figures: diagram of distribution of collections in the two types of habitats: natural (forest stream) and flow-limited pond; Bottom figure: concavity of the recipient used to capture mosquito immature stages and collector position in the larval habitat, (3×) three intercalate collections at each point.

Source:SVS/MS, 2011.

Appendix F.

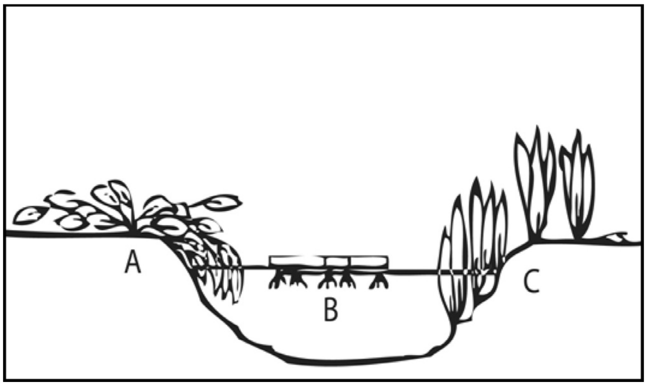

Fig. F1. Schematic representation of the refuge types associated in the water level: (A): Submerged; (B): Floating; (C): Emerging. The category Bank means that there is no associated vegetation.

Appendix H.

Fig. H1. Distribution of L1 larvae of *Anopheles* sp. by type of breeding-site, total of subunits (n = 595), Remansinho settlement, Lábrea, Amazonas, Brazil.

Appendix G.

Table G1
Mean and Standart Desviation (Std.Dev.) to physical-chemical variables (ph, temperature in °C, conductivity in μS/cm and dissolved oxygen in mg/L) for each landscape' breeding sites in the study period, Remansinho settlement, Lábrea, Amazonas, Brazil.

Breeding-site		May/2012				April/2013				May/2013			
		ph	temp	cond	oxig	ph	temp	cond	oxig	ph	temp	cond	oxig
Natural													
1	Mean	5.28	25.03	9.18	2.47	5.89	25.39	6.86	1.91	6.03	25.62	8.14	2.16
	Std.Dev.	0.42	0.45	1.42	0.93	0.30	0.60	0.44	0.46	0.48	0.713	1.21	0.60
2	Mean	4.89	24.72	8.11	2.76	5.92	25.46	9.89	2.09	5.81	25.25	10.53	3.03
	Std.Dev.	0.33	0.74	2.54	1.14	0.41	0.53	1.08	0.79	0.62	0.77	2.39	0.69
3	Mean	5.22	24.77	10.09	4.22	5.53	25.31	9.87	3.43	5.99	24.89	8.97	3.64
	Std.Dev.	0.46	0.41	1.53	1.30	0.42	0.83	2.14	1.59	0.49	0.37	2.29	1.78
4	Mean	5.56	24.99	8.96	2.63	5.84	25.93	7.87	4.13	5.31	25.80	8.61	1.61
	Std.Dev.	0.47	0.45	2.12	0.66	0.57	0.50	1.66	0.79	0.52	0.934	1.51	0.40
Man-made													
5	Mean	6.53	26.19	9.93	3.18	7.12	27.95	12.26	2.94	6.79	27.91	17.34	4.58
	Std.Dev.	0.26	0.18	1.37	0.65	0.245	0.49	1.48	0.95	0.441	1.33	1.959	0.72
6	Mean	6.03	25.81	12.27	3.05	6.93	28.30	19.63	3.98	6.57	28.24	17.31	5.90
	Std.Dev.	0.31	0.83	3.87	0.77	0.359	0.72	2.71	1.11	0.514	0.91	2.64	1.17
7	Mean	6.56	27.69	8.56	4.32	7.08	29.35	6.35	5.03	6.665	28.44	17.03	4.87
	Std.Dev.	0.40	0.62	1.06	0.71	0.57	0.65	0.65	0.41	0.63	1.07	2.01	0.49
8	Mean	6.33	26.78	6.53	3.94	7.15	29.14	11.78	2.78	6.79	29.57	13.34	4.15
	Std.Dev.	0.371	0.29	3.08	1.20	0.283	0.99	1.59	0.81	0.489	1.11	2.72	1.15

Appendix I.

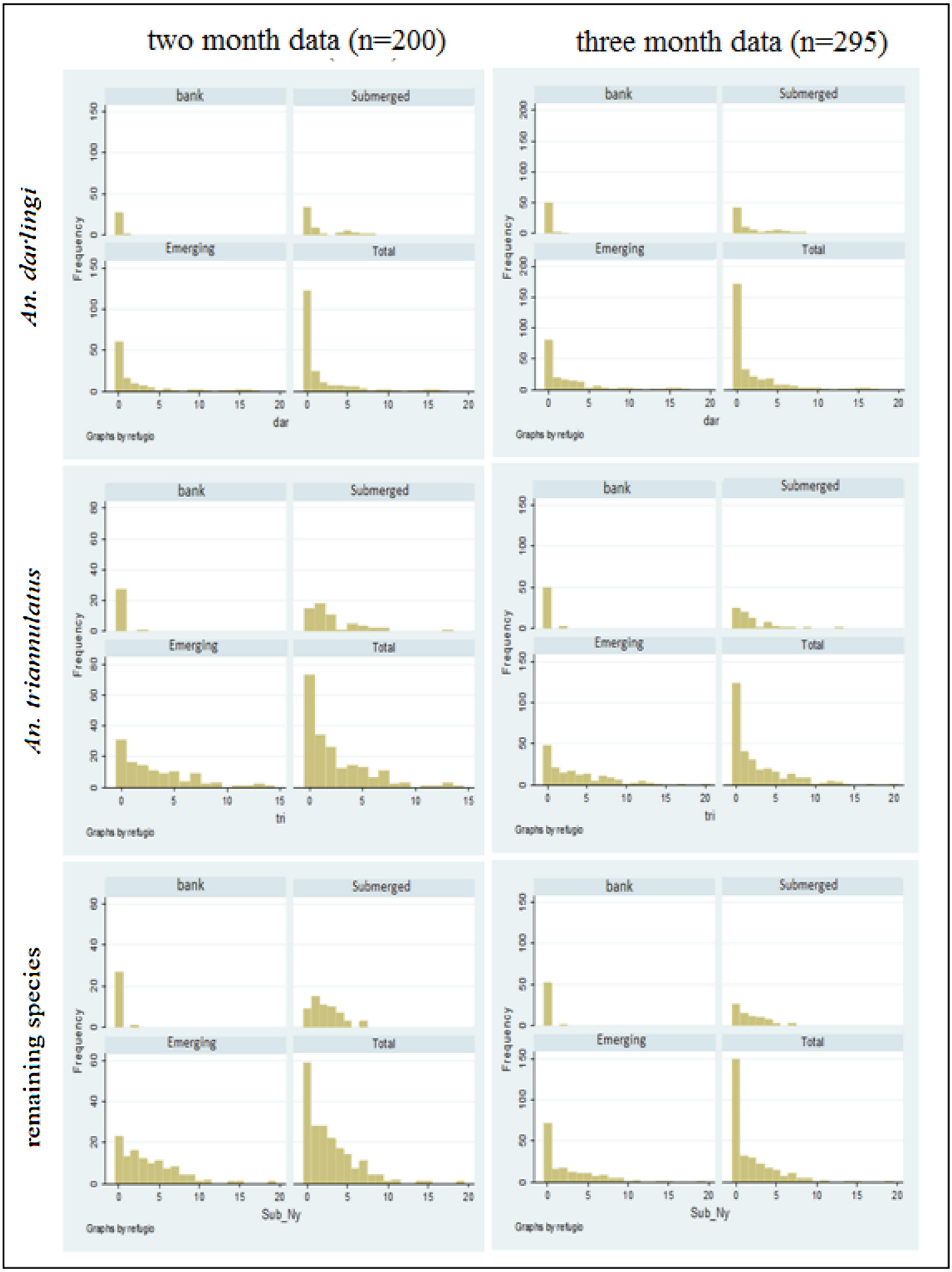

Fig. I1. Histograms for the taxonomic groups, by class of categorical variable refuge (bank, submerged and emerging) and sample size approaches (n = 295 and n = 300), Remansinho settlement, Lábrea, Amazonas, Brazil.

Appendix J.

Table J1
Pearson's chi-squared test colinearity between the physical-chemical variables and *Nyssorhynchus* taxonomic groups for sample size approaches (n = 295 and n = 300), Remansinho settlement, Lábrea, Amazonas, Brazil.

sample size		*An. darlingi*	*An. triannulatus*	remaining sp.	pH	Temperature	Conductivity	Dissolv. O2
three-month data (n = 295)	*An. darlingi*	1						
	An. triannulatus	−0.043	1					
	remaining sp.	0.039	0.290	1				
	pH	−0.079	−0.030	0.266	1			
	Temperature	0.054	−0.236	0.171	0.407	1		
	Conductivity	0.068	0.139	0.278	0.092	0.154	1	
	Dissolv. O2	0.075	−0.075	−0.062	0.049	0.230	0.130	1
two-month data (n = 200)	*An. darlingi*	1						
	An. triannulatus	−0.137	1					
	remaining sp.	0.041	0.494	1				
	pH	−0.172	0.072	0.077	1			
	Temperature	0.057	−0.249	−0.243	0.052	1		
	Conductivity	0.106	0.103	0.067	−0.233	−0.320	1	
	Dissolv. O2	0.089	0.038	−0.226	−0.178	0.021	0.075	1

Appendix K.

Table K1
Adjustment indicators for Negative Binomial Regression, for insertion of the independent variables (All and Cut-off), model (usual and zero-inflated) and sample size approaches (n = 295 and n = 300), Remansinho settlement, Lábrea, Amazonas, Brazil.

Groups		two-month data (n = 200)				three-month data (n = 295)			
		Negative Binomial		Negative Binomial Zero Inflated		Negative Binomial		Negative Binomial Zero Inflated	
		All variables	Cut-off uni-variables	All variables	Cut-off uni-variables	All variables	Cut-off uni-variables	All variables	Cut-off univariables
An. (Nys.) darlingi	Deviance	593.988 (192)	604.626 (194)	591.155 (191)	604.879 (193)	907.619 (286)	912.642 (288)	906.523 (285)	911.726 (287)
	AIC*n:	609.988	616.626	609.155	618.879	925.619	926.642	926.523	927.726
An. (Nys.) triannulatus	Deviance	742.769 (192)	745.185 (193)	742.24 (191)	746.525(192)	1088.581 (286)	1090.794 (287)	1092.667 (285)	1097.812 (286)
	AIC*n:	758.769	759.185	760.24	762.525	1.106.581	1.106.794	1.112.667	1.115.812
Remaining sp.	Deviance	773.410 (192)	786.043 (193)	778.290 (191)	787.197 (192)	898.300 (286)	904.967 (287)	903.414 (285)	908.525 (286)
	AIC*n:	789.410	800.043	796.290	803.197	916.300	920.967	923.414	926.525

Values in brackets for deviance: (degrees of freedom).

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