

1 **Tree hole mosquito species composition and relative abundances**

2 **differ between urban and adjacent forest habitats in northwestern**

3 **Argentina**

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25 **Running head: treehole mosquito fauna**

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Abstract

28 **Water** holding tree holes are main **larval habitats** for many pathogen vectors, especially
29 mosquitoes (Diptera: Culicidae). Along three years, the diversity **and composition** of mosquito
30 species in tree holes of two neighbouring but completely different environments, a city and its
31 adjacent forest, were compared **using generalized linear mixed models, PERMANOVA,**
32 **SIMPER and species association indexes.** The city area (Northwest Argentina) is highly relevant
33 epidemiologically due to the presence of *Aedes aegypti* L. (main dengue vector) and occurrence
34 of dengue outbreaks; the Yungas rainforests are highly biologically diverse. In total 7 mosquito
35 species were recorded, in descending order of abundance: *Ae. aegypti*, *Haemagogus spegazzinii*
36 Brèthes, *Sabethes purpureus* (Theobald), *Toxorhynchites guadeloupensis* Dyar and Knab, *Aedes*
37 *terrens* Walker, *Haemagogus leucocelaenus* Dyar & Shannon and *Sabethes petrocchiaie*
38 (Shannon and Del Ponte). The 7 mosquito species were recorded in both city sites and forested
39 areas; however, their mosquito communities significantly diverged because of marked
40 differences in the frequency and relative abundance of some species: *Tx. guadeloupensis* and *Ae.*
41 *aegypti* were significantly more abundant in forest and urban areas, respectively. **Positive**
42 **significant associations were detected between *Ae. aegypti*, *Hg. spegazzini* and *Hg.***
43 ***leucocelaenus*.** The combined presence of *Ae. aegypti*, *Haemagogus* and *Sabethes* in the area
44 also highlight a potential risk of yellow fever epidemics. **Overall results show an impoverished**
45 **tree hole mosquito fauna in urban environments, reflecting negative effects of urbanization on**
46 **mosquito diversity.**

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48 **Keywords:** Culicidae, ecology, interspecific association, phytotelmata, vector, Yungas
49 rainforest.

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Introduction

53 Tree holes are among the most abundant phytotelmata (i.e., plant-held waters; Kitching, 2000) in
54 many tropical and temperate forests. A variety of macro-organisms use these natural cavities as
55 larval habitat and many species develop exclusively in these sites. Since they are main container
56 larval environments for many pathogen vectors, especially mosquitoes (Diptera: Culicidae), tree
57 holes may be medically and economically important habitats (Yanoviak, 2001). *Aedes* Meigen
58 mosquitoes that transmit both dengue, chikungunya and zika viruses are known to use tree holes
59 as larval habitat in India (Selvan *et al.*, 2016) and in tropical rainforests of Nigeria, Africa
60 (Anosike *et al.*, 2007). *Aedes aegypti* L., the main global vector of dengue and urban yellow
61 fever (Gubler, 2004) is usually linked to artificial containers, but immature stages have also been
62 collected from tree holes in different parts of the world (Yadav *et al.*, 1997, Marquetti *et al.*,
63 2005, Anosike *et al.*, 2007, Hribar & Whiteside, 2010) including recently in Argentina, Salta
64 province (Mangudo *et al.*, 2011; Mangudo *et al.*, 2015). *Aedes triseriatus* (Say) is a common
65 North American tree hole mosquito which is regularly found cohabiting with predatory larvae of
66 *Toxorynchites rutilus* (Coquillett) in the southern USA (Lounibos *et al.*, 1993), and was also
67 reported competing with the invasive species *Ae. albopictus* (Skuse) (Lounibos *et al.*, 2001).
68 Species of *Haemagogus* Williston are of great interest in South America because of their role in
69 sylvatic yellow fever transmission and some species of *Sabethes* Robineau-Desvoidy are known

70 to harbour and transmit this and other arboviruses (Degallier *et al.*, 1992). Even though early
71 researches recorded and attempted to control the vectors or suspected vectors of yellow fever in
72 tree holes (and other phytotelmata), still little is known about their bionomics.

73 Mosquito species diversity may indirectly alter transmission risk, for example by limiting
74 mosquito abundance (including vector abundance) since any factor that reduces vector
75 abundance is expected to decrease the entomological risk of disease transmission (Chaves *et al.*,
76 2011). Moreover, as more species cohabit, more interspecific interactions between vector and
77 non-vector species are possible, which may affect vector capacity. It has been shown for
78 example, that an increase in larval competition between *Ae. aegypti* and *Ae. albopictus* enhanced
79 the proportion of the latter that transmits dengue virus (Alto *et al.*, 2008). In other studies it was
80 observed that the presence of *Ae. albopictus* reduced the survival of *Ae. triseriatus* larvae (vector
81 of La Crosse virus in USA), but in turn, from the surviving larvae emerged bigger female
82 mosquitoes that were more given to develop disseminated virus infections, thus affecting the
83 pathogen transmission risk (Bevins *et al.*, 2008).

84 Culicids are being found in micro-ecological habitats differing from their known ecological
85 habitats, such as the collection of *Anopheles gambiae* ss (Omlin *et al.*, 2007) and *An. funestus*,
86 Giles typically found in ground ponds, in tree holes in Nigeria, Africa (Anosike *et al.*, 2007). The
87 presence of sylvan species in urban areas may be relevant if these species are or become
88 involved in arbovirus transmission. We recently found larvae and pupae of *Sabethes purpureus*
89 (Theobald) in tree holes both in urban and forest environments in Orán, Salta province
90 (Argentina). Although this species is commonly assumed to be mostly restricted to the forest
91 habitat, a higher proportion of tree holes was positive in the urban compared to the forest
92 environment (Mangudo *et al.*, 2014).

93 The aims of this study were to further characterize the mosquito fauna developing in tree holes in
94 the Yungas rainforests of Salta, Argentina, and to assess whether species composition and
95 diversity were similar between urban (San Ramón de la Nueva Orán city) and adjacent forested
96 areas. This region is located within an area of high biological diversity (Malizia *et al.*, 2012;
97 Szumik *et al.*, 2012), combined with epidemiological potential due to the presence of *Ae.*
98 *aegypti*, the occurrence of dengue outbreaks and the recent introduction of Chikungunya virus
99 (Pan American Health Organization, 2017). Moreover, several provincial departments of Salta
100 within Yungas rainforest, including Oran, are considered as potential risk areas for Yellow Fever
101 by the World Health Organization (WHO, 2014). They harbour a cebus monkey (*Cebus capella*)
102 potentially capable of supporting YF infection. These characteristics make the region an
103 interesting model to study tree hole culicid communities and eventually their relation to the
104 epidemiology of mosquito-borne diseases.

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Materials and methods

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Study area

108 San Ramon de la Nueva Orán, hereafter Orán, is a city in northwest Argentina (23°08'S,
109 64°20'W, elevation 337 m.a.s.l.; Fig. 1). Covering an area of 20 km², it is the second largest
110 urban centre of Salta province with a population of approximately 82000 (INDEC, 2010). Orán
111 is located within the Pedemontane rain-forest floor of the Yungas, where jungles of
112 *Calycophyllum multiflorum* Griseb (Rubiales: Rubiaceae) and *Phyllostylon rhamnoides*
113 (Poison) Taub. (Urticales: Ulmaceae) and vines predominate (Brown *et al.*, 2001). The area has
114 been subjected to changes related to human activities (mainly urbanization, industrial
115 development, agriculture, and forestry). The climate is subtropical, with an average summer

116 temperature of 27.7° C and winter temperature of 16.4° C. The mean annual rainfall is 1000 mm,
117 occurring mostly during the warmer months (October to April).

118 The city is characterized by a densely built central area where houses with small or no front
119 yards predominate and there are few low buildings. Suburban areas have a lower building
120 density with bigger gardens, more trees and are closer to the border with the seminatural region.
121 Native Yungas forest remains to the east, north-east and south-east of the city, while the western
122 and southern regions are mostly agricultural (INTA, 2004).

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Tree hole selection

126 Tree holes were inspected in 1,213 trees in the main forested areas of the city, including parks,
127 sidewalks and areas within the graveyard and the sports centre, as described in Mangudo *et al.*
128 (2014). Briefly, trees were examined to assess whether they bore holes up to 2 m above the
129 ground that could hold water. Additionally, trees were inspected within a square area (50 x 50
130 m²) at three Yungas forest sites located at 0.5 km to the northeast, 7 km to the east and 4 km to
131 the southeast from the urban border. The forested areas were connected to downtown by rural
132 paths. Each tree was assigned to one of three habitat categories according to its location and
133 urbanization degree: urban (central area of the city, with a mean density of 81 homes per ha, and
134 at least 40% built surface (including roads)), suburban area (peripheral area of the city, with a
135 mean density of 23 homes per ha and 20% or less built surface) and forest (out of the city,
136 without buildings). Tree species and the presence or absence of water holding holes, as well as
137 location of trees with holes (Garmin global positioning system, Garmin eTrex Legend, Olathe,
138 KS) were recorded.

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Entomological sampling

142 Sixty three (5%) of the trees examined had 69 holes that held water. Tree holes types included

143 pans formed as branch intersection and rot holes (Mangudo *et al.*, 2015). The presence and

144 volume of water and number of immature stages (larvae and/or pupae) in each of these tree holes

145 was recorded approximately once a month from January to April of 2011 and from January to

146 March of 2012 and 2013. The time frames comprise the season when rainfall is most likely to

147 flood the tree holes, based on previous (informal) observations, the capability to retain water

148 could not be sustained during the winter dry months. The average monthly rainfall of the

149 sampling season was 135 ± 13 mm. Within a given month, inspecting all water holding tree holes

150 took three to five consecutive days. At a sampling date all water content was removed from the

151 tree hole using a siphon bottle, and thus the larvae found during the following sampling date

152 belonged to new cohorts (Mangudo *et al.*, 2014, 2015). Water contents were examined on site in

153 a white plastic tray to separate potential predators and specimens were transported to the

154 laboratory for further processing. Larvae of the 1st, 2nd and 3rd instars and pupae were reared

155 either to the 4th instar or to adult emergence. Taxonomic determinations were based on

156 morphological characteristics of 4th instar larvae and/or adults, using Darsie (1985) and Forattini

157 (1996, 2002) keys and species descriptions. Abbreviations for mosquito genera and subgenera

158 are those proposed by Reinert (2009).

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Data analysis

161 The set of Culicidae collected in a tree hole on a sampling date was considered as a sample. To
162 assess completeness of the mosquito inventory, the sample coverage C , i.e. the total probability
163 of occurrence of the species observed in the reference sample (Chao & Shen 2010, Chao & Jost
164 2012), was estimated for the community from each habitat category considering the pooled
165 samples from a habitat. Since mosquitoes lay their eggs in batches, and thus larvae of a species
166 are usually clumped in space, abundance-based rarefaction may underestimate species richness.
167 Thus, instead of species frequencies, incidences (i.e., the number of positive samples for a
168 species) were considered for these analyses as recommended by Gotelli and Chao (2013).

169 To compare overall species richness of the three habitats, a species accumulation curve was
170 estimated for each habitat using sample interpolation (rarefaction) and extrapolation (to less than
171 twice the number of samples recorded in a site) from the total number of samples collected on
172 each habitat, using the multinomial model (Colwell *et al.*, 2012) provided by EstimateS software
173 (Colwell, 2013) (i.e., the expected number of species represented in m samples, given a reference
174 sample). The option to randomize samples with replacement was selected, whose advantage is
175 that the variance of the estimators is unconditional to the accumulation of samples.

176 Species diversity per tree hole and sampling date was estimated as the observed number of
177 species (richness, S ; diversity of order zero) and computing the effective number of species (or
178 diversity of order one; see, for example, Jost 2006).

$$179 \quad {}^1D = \exp(-\sum_{i=1}^S p_i \ln p_i) \quad (1)$$

180 where p_i (importance value) = n_i/N , N = total abundance of all species, n_i = abundance of species
181 i , and $\ln()$ the natural logarithm.

182 Generalized linear mixed models (GLMM) (Infostat; Di Rienzo *et al.*, 2014) were used to assess
183 if mosquito diversity per tree hole significantly differed between habitats. Dependent variables
184 were the effective number of species and richness per tree hole per sampling date (assuming a

185 Poisson distribution), fixed effect was habitat (urban, suburban and forest) and random effects
186 were month and tree hole. To evaluate if diversity was significantly different between tree hole
187 type (branch intersection and rot hole) and habitat GLMM (Infostat; Di Rienzo *et al.*, 2014) were
188 also used; tree hole was the random effect.

189 To assess if there were differences in species composition between habitats (urban, suburban and
190 forest) a non-parametric multivariate analysis of variance (PERMANOVA) with 10000
191 permutations based on Bray-Curtis distances was used. The total of specimens from each species
192 collected in each tree hole per sampling date (data transformed to $\ln(n+1)$) was considered a
193 sample. To determine which species contributed more to dissimilarity among tree holes for those
194 pairs of habitats that showed significant differences on previous analysis, a SIMPER method
195 (similarity percentage) was used; it is available in Past software (Hammer *et al.*, 2001).

196 Since no significant differences were found in species composition between urban and suburban
197 habitats, those data were regrouped in a “city” category in order to explore if associations of
198 pairs of species were similar in comparison with the forest. Significance of species associations
199 (C_{AB}) were analysed using Cole’s C7 index (1949) by 2x2 contingency tables and χ^2 (Hurlbert
200 1969). At each sampling date, all water content (and larvae+pupae) was extracted from the tree
201 hole, therefore immature found on two consecutive sampling dates belong to different cohorts
202 due to instalment hatching from different flood events or colonization events. Only those dates
203 when both species of a pair were present were considered in order to reduce seasonal segregation
204 effects; furthermore, from this data pool, species detected on less than 5 different trees were
205 excluded from the analysis to reduce samples dependence on site.

206 Finally, Spearman r_s nonparametric correlation was used to assess if abundance and species
207 diversity were related with water volume. For all tests, a P-value < 0.05 was considered to

208 represent significant differences. Throughout the text, the results are presented as the mean
209 plus/minus the standard error.

210

211 **Results**

212 We collected 5,498 immature culicids in 26 species of trees from a total of 170 samples,
213 belonging to 7 species from 5 genera, in descending order of abundance: *Ae. aegypti*,
214 *Haemagogus spegazzinii* Brèthes, *Sa. purpureus*, *Toxorhynchites guadaloupeensis* Dyar and
215 Knab, *Aedes terreus* (Walker), *Haemagogus leucocelaenus* Dyar & Shannon and *Sabethes*
216 *petrocchiaie* (Shannon and Del Ponte) (Table 1). In urban and suburban environments, *Ae.*
217 *aegypti* was the most frequent species, no specimen of *Ae. terreus* or *Sa. petrocchiaie* were
218 detected in urban tree holes while *Hg. leucocelaenus* was not found in the suburban samples. All
219 species were found in the forest, although *Ae. aegypti* occurrence was an exceptional event in
220 this habitat, in a tree hole within its flight dispersal range from the city border (Mangudo *et al.*,
221 2015).

222 Sampling efficiency (estimated sample coverage) was estimated to be 0.98 or higher for each
223 habitat, while rare species coverage was 0.95 or higher for all habitats (Table 1). Using the
224 method of Shen *et al.* (2002), less than one additional species was predicted in a further survey of
225 100 samples from urban (0±0), suburban (0.3± 0.5) or forest (0.4±0.7) habitats. Taken together,
226 the results of the present study indicate that the samples obtained provide adequate
227 representation of the species diversity at all three habitats.

228 Results of rarefaction-extrapolation curves were compared among the three habitats considering
229 50 samples, which was less than the double of the smaller number of samples collected in any of
230 the three habitats (following Colwell's rules; Colwell *et al.* (2012)) (Fig. 2). The 95%

231 unconditional confidence intervals of urban and suburban tree holes did not overlap, indicating
232 that mosquito richness in urban habitat was significantly lower than in the suburban habitat. On
233 the other hand, the confidence intervals of forest richness fully overlapped with those of urban
234 and suburban habitats. Following the conservative rule of overlapping intervals proposed by
235 Colwell *et al.* (2012), we inferred the overall richness did not significantly differ between the
236 forest and urban habitats or between forest and suburban habitats. However, when considering
237 the species relative abundances, the effective number of species of forest was 3.6 and 4.5 times
238 higher than the urban and suburban environments, respectively (Table 1).

239 The richness (S) per tree hole (sample), on the other hand, was relatively low (1 species in 72 %
240 of the samples, and up to 4 species in the remaining 28 %) and no significant effect of habitat
241 type on this variable was detected ($p = 0.72$). Consistently, the effective number of species per
242 tree hole was also low (ranging from 1.22 to 1.43 species) and did not differ among habitats ($p =$
243 0.69) (Table 2). Significant correlations between diversity and water volume were not detected
244 either (Spearman $r_s = 0.10$, $P = 0.17$). On the other hand, there was a positive and significant
245 correlation between larval abundance and water volume (Spearman $r_s = 0.36$, $P < 0.0001$). Mean
246 water volume recorded was 239.8 ± 46.4 ml and ranged from 10 to 3450 ml.

247 Significant differences in species composition among habitats were found with PERMANOVA
248 ($F = 37.83$; $P = 0.0001$), being the forest different from urban and suburban environments ($P =$
249 0.0001); tree holes from urban and suburban environments did not differ significantly ($P = 0.33$)
250 in their species composition. The SIMPER analysis indicated that differences among tree holes
251 in the city (grouping urban and suburban tree holes) and forest were mainly due to the higher
252 abundance of *Ae. aegypti* (contributing 49.07% to the dissimilarity) and to a lower abundance of
253 *Tx. guadaloupenis* (contributing 17.07%) in the city tree holes (Table 3).

254 In the urban environment, in approximately half the tree holes *Ae. aegypti* was always collected
255 as a single species (Table 4). A similar pattern was observed for *Tx. guadaloupenensis* in the forest.
256 In contrast, in most trees holes *Hg. leucocelaenus*, *Hg. spegazzini* and *Sa. purpureus* were
257 collected at least once coexisting with other species. Table 5 shows the number of samples each
258 pair of species was collected together. Based on the C7 coefficient of association of Cole (1949),
259 positive significant associations were detected between *Ae. aegypti*-*Hg. leucocelaenus* ($0.23 \pm$
260 0.09 ; coefficient of association \pm standard error), *Ae. aegypti*-*Hg. spegazzini* (0.15 ± 0.05), and
261 *Hg. spegazzini*-*Hg. leucocelaenus* (0.53 ± 0.14 in the city and 0.48 ± 0.16 in the forest).
262 Although *Tx. guadaloupenensis* was less frequently found coexisting with other species (Table 6),
263 the coefficients that could be assessed were not significant (-0.24 ± 0.36 with *Ae. aegypti*; -0.05
264 ± 0.29 with *Hg. spegazzini*).

265

266 **Discussion and conclusions**

267 There is a growing interest in studying mosquito communities because of the potential influence
268 of species diversity on pathogen cycles and transmission. In the present study, *Ae. aegypti* was
269 widely detected in tree holes throughout the city and was even predominant over other culicids
270 collected during the three years long research, being found positively associated with *Hg.*
271 *leucocelaenus* and *Hg. spegazzini*. The presence of *Ae. aegypti* in tree holes may be relevant
272 especially in cities like Orán, where small outbreaks of dengue are common and high densities of
273 this vector increase the risk of large epidemics as the one observed in 2009 which affected 18
274 provinces and about 26,000 people (Torres, 2010) or in 2016 with more than 76,900 cases
275 (Ministerio de Salud, 2016). As has already been discussed in Mangudo *et al.* (2015), our only
276 record of *Ae. aegypti* in the forest was made in a partially disturbed natural area, close to the

277 urban border (300 m), and occasionally visited by people through a rural path connecting with
278 the city. Thus, *Ae. aegypti* occurs in close association with human populations and despite the
279 availability of larval habitats, it does not seem to establish in the forest.

280 Urbanization is one of the most severe land uses, causing significant changes in the environment,
281 creating a mosaic of patches with different types of coverage (Alberti *et al.*, 2004). In Brazil,
282 mosquito species richness and diversity increased from lower to higher forest cover and was
283 highest in sites with highest diversity and number of larval habitats (Correa *et al.*, 2014).
284 Similarly, in Kenya, Africa, a significantly lower mean number of mosquito species per sample
285 was recorded in a peridomestic zone compared to two forested habitats (Lounibos, 1981). In the
286 present study we observed lower global species richness in the urban compared to the suburban
287 environment. However, although the total number of species observed in the forest was higher
288 than in the city, there were no significant differences in richness in the forest compared to urban
289 and suburban environments, which may be related to the sporadic occurrence of *Ae. aegypti* and
290 *Sa. purpureus* in the forest. On the other hand, there was a higher effective number of species in
291 the forest compared to the urban or suburban sites, explained by the higher abundance and
292 dominance of *Ae. aegypti* in the city compared to the forest tree holes. The higher richness we
293 found in the suburban habitats compared to urban, a transition area between the urban and forest
294 environments, may reflect the fact that some culicid species adapted to wild or human
295 environments could find in the suburban sites intermediate conditions they tolerate.
296 Alternatively, species from wild areas could be spilling over to the suburban areas, which could
297 act as a sink habitat. These diversity patterns were also found in other systems. For example,
298 Tóthmérész *et al.* (2011) studied diversity of carabid beetles along a rural–urban gradient
299 representing increasing human disturbance. They found that the total number of carabid species

300 was significantly higher in the suburban area followed by the rural area and was the lowest in the
301 urban area. Their results did not support what they expected, that is, an increase in disturbance
302 would **monotonically** decrease diversity. The overall impact of urbanization is different on
303 different species; it depends for example on species habitat affinities (forest specialists or
304 generalists) (Tóthmérész *et al.*, 2011).

305 **When assessing local (sample) species richness or diversity, i.e., per tree hole and date, no**
306 **significant differences were detected on richness or relative abundances (expressed as effective**
307 **number of species). This could be due to the low number of species that coexists in the same tree**
308 **hole in a given date, which typically ranged from 1 to 3 species.**

309 We found significant differences on species composition between tree holes located in forested
310 environments and urban or suburban tree holes. These differences were mainly due to relative
311 abundances and/or frequencies of *Ae. aegypti* and *Tx. guadaloupenis* (Table 4), being the former
312 more abundant in urban and suburban environments **(and almost absent from the forest)** and the
313 latter more frequent in the forest.

314 The genus *Toxorhynchites* includes mostly tropical mosquitoes; species of *Toxorhynchites* are
315 usually found more frequently and in higher abundances in vegetated zones (Corbet, 1964;
316 Lounibos, 1981). However, it must be noted that in Orán the urban specimens were collected
317 from tree holes located both in the centre and in the western border of the city (3 and 5 km
318 approximately from the forest, respectively), and no specimens were obtained from urban tree
319 holes closer to the eastern forested region. Thus, finding these larvae and pupae in tree holes in
320 the city would more likely indicate the species actually dwelling in this urban environment,
321 instead of sporadic occurrence from forest dispersing individuals.

322 The predatory genus *Toxorhynchites* is of particular interest within tree hole mosquitoes as it
323 exploits a different niche than most other tree hole mosquitoes (which are browsers and filter
324 feeders) (Srivastava, 2005). In the present study, both in the forest and in city areas *Tx.*
325 *guadaloupenis* was most frequently collected as a single species (67% of the samples). All
326 members of the genus *Toxorhynchites* are obligate predators (Lounibos, 1981) and since the
327 other culicid species are potential prey for *Tx. guadeloupenis*, these results may be a direct
328 consequence of predation. On the other hand, they could be in some extent the result of
329 oviposition preferences, since the oviposition behavior of adult mosquitoes is responsible for the
330 spatial occurrence of larvae (Lounibos, 1981). Further studies are needed to confirm these
331 hypotheses.

332 Some studies related the presence of *Toxorhynchites* with more diverse mosquito communities in
333 forested environments (Corbet, 1964). Probably, this predator contributes to prey coexistence by
334 lowering their densities and alleviating their interspecific competition (Mogi & Young, 1992) or
335 by selective feeding on prey species (Bradshaw & Holzapfel, 1983). However, a study in Kenya
336 showed higher abundances of *Tx. brevialpis* Theobald in ecotonal and forested zones, which
337 yielded the highest species richness values, but found no significant correlation between number
338 of prey species per sample and the presence of the predator (Lounibos, 1979). Consistently, in
339 our study *Tx. guadaloupenis* was found only in 28% of the samples holding more than two
340 species. Moreover, the effects of *Toxorhynchites* on potential prey diversity observed in some
341 studies have been opposed and could partially depend on phytotelmata species. The presence of
342 *Tx. haemorrhoidalis* (Fabricius) significantly reduced the mean number of taxa of potential prey
343 in *Heliconia* spp., but no significant effect on taxon richness was observed among bamboo
344 samples containing this predator (Lounibos *et al.*, 1987). Even though at tree hole level

345 community diversity (estimated as richness or effective number of species) was similar, there
346 were overall differences in species composition between urbanised environments (urban and
347 suburban) and the forest; whether diversity patterns in tree holes in this region may be related to
348 the presence of *Tx. guadaloupenensis* is not clear. Besides affecting species diversity, predators
349 such as *Toxorhynchites* that feed on vector mosquitoes may influence on their abundances and
350 indirectly in their vector capacity (Lounibos & Campos, 2002). We can speculate that the
351 negative association (albeit non-significant) between *Tx. guadaloupenensis* and *Ae. aegypti* could
352 indicate (at least in tree holes) that the first species may exercise partial control over the second.
353 However, effects of predators on mosquito and their consequences for populations and for
354 mosquito-borne disease are not fully understood (Juliano, 2009).

355 In a previous related work on *Sa. purpureus*, a species considered sylvatic and whose larval
356 habitats are poorly known, we extended its known geographic distribution (Mangudo *et al.*,
357 2014). Finding this species in Orán region, and particularly in urban environments, is interesting
358 for many reasons. On the one hand, the species has been collected in essentially sylvatic biotopes
359 (Guimarães *et al.*, 2003; Cardoso *et al.*, 2005; D'Oría *et al.*, 2010) and in forest patches within
360 urban settings (Reis *et al.*, 2010), suggesting that it was mostly restricted to the forest habitat.
361 The fact that larvae and pupae were collected from water-holding tree holes, both in the urban
362 and suburban environments, suggests that in Orán immature stages of *Sa. purpureus* complete
363 their development in these larval habitats, and thus environmental conditions are adequate for
364 this species to breed within an urban landscape; or its niche breadth could be wider than the
365 assumed.

366 Consistently with the fact that positive associations between species are relatively frequent
367 among non-predatory mosquitoes in container habitat such as tree holes (Sota *et al.*, 1994),

368 significant associations detected in this study were positive. We found both *Hg. leucocelaenus*
369 and *Hg. spegazzini* positively associated with *Ae. aegypti* in the city. *Haemagogus leucocelaenus*
370 was positively associated with *Hg. spegazzinii* regardless of the habitat where the tree hole was
371 located. In contrast, in tree holes in a sylvan habitat in Chaco province, Argentina, *Hg.*
372 *spegazzinii* showed the lowest values of temporal niche superposition with *Hg. leucocelaenus*
373 (Stein *et al.*, 2011). In the Atlantic forest of Rio de Janeiro, Brazil, *Hg. leucocelaenus* did not
374 show significant associations with other species ovipositing at similar heights, but rather tree
375 height segregation was considered a strategy to reduce interspecific competition (Alencar *et al.*,
376 2016. Instalment hatching (*i.e.*, variable egg hatch in response to flooding), a bet-hedging
377 strategy that allows some larvae to complete development in transient environments, may
378 temporally segregate species within a tree hole (Lounibos, 1981). However, significant positive
379 associations were detected between *Aedes* and *Haemagogus* species showing this behaviour
380 (Lounibos, 1981, Alencar *et al.*, 2014) suggesting that resource competition may not be a
381 limiting factor in this community. Since *Aedes* mainly exhibit shredding or collecting-filtering
382 feeding mode, while *Haemagogus* are collector-gatherers (Merritt *et al.*, 1992), food resource
383 partitioning may explain coexistence.

384 *Haemagogus* species have great interest due to their role on sylvan yellow fever transmission in
385 South America; particularly *Hg. spegazzinii* has been collected mainly from tree holes in high
386 abundances in forested areas in Brazil and northeast of Argentina, becoming relevant as potential
387 vector of sylvan yellow fever in the region (Stein *et al.*, 2011). *Haemagogus leucocelaenus* is
388 considered a secondary yellow fever vector in some regions (Monath, 1988); however its role as
389 the main yellow fever vector was confirmed in southern Brazil (Cardoso *et al.*, 2010). The
390 arboviruses Wyeomyia, Ilhéus, Maguari, Tucunduba and Una virus have also been isolated from

391 this species (Karabatsos, 1985; Hervé *et al.*, 1986). *Haemagogus leucocelaenus* was suspected of
392 transmission in outbreaks in Bolivia and Argentina (Monath, 1988) and has received attention
393 due to its increasing medical importance (Forattini & Gomes, 1988). As of December 2016,
394 Argentina formally requested WHO that the International travel and health (ITH) text on that
395 country should be changed to state that yellow fever vaccination is recommended only for
396 travellers to the provinces of Corrientes and Misiones. ([http://www.who.int/ith/yellow-fever-](http://www.who.int/ith/yellow-fever-risk-mapping/3rd-GRYF-meeting-report.pdf?ua=1)
397 [risk-mapping/3rd-GRYF-meeting-report.pdf?ua=1](http://www.who.int/ith/yellow-fever-risk-mapping/3rd-GRYF-meeting-report.pdf?ua=1)). However, the current 2017 outbreak of
398 Yellow fever in neighboring Brazil has expanded to areas not considered as with risk. It is
399 speculated that changes in viral proteins may be involved in this unusual event, as they may
400 impact the capacity of viral infection in vertebrate and/or invertebrate hosts (Bonaldo *et al.*,
401 2017). Hence the relevance of the present record of immature stages in tree holes in Oran city
402 and adjacent forested areas.

403 Regarding tree hole features that may influence mosquito fauna, we found that the number of
404 culicid individuals in tree holes in Orán was significantly and positively correlated to water
405 volume, as has been described for tree holes in Panama (Yanoviak, 1999). Since water volume
406 contained in a tree hole can be used as a measure of its size (Yanoviak, 1999), larger water
407 volume would indicate larger tree holes; and probably larger tree holes are less prone to
408 dehydration, retaining water longer than harbouring bigger mosquito communities. Several
409 studies had shown positive correlations of species richness and/or abundance with tree hole
410 volume (Lounibos, 1981; Sota *et al.*, 1994). Tree holes contain relatively few mosquito species
411 on average (usually less than three) and Srivastava (2005) suggested that this would be not
412 because of competitive exclusion (i.e. saturation) but rather due to the high rate of drought
413 disturbance in the system. This could be one reason why tree holes in Orán harboured low (less

414 than two) average number of culicid species per sample both in forest and urban areas, since as
415 they were small could be more prone to dehydration. Our results agree with average local (tree
416 hole level) species richness values recorded in other studies; Lounibos (1981) reported similar
417 mean number of tree hole mosquito species per sample in two African forests (1.6 to 2.4,
418 excluding root buttresses, not considered in our study) and in a survey in southwestern Japan, the
419 median number of species per tree hole was two (Sota *et al.*, 1994).

420 Culicids productivity from tree holes is usually low compared to artificial containers such as
421 used tires (Burkot *et al.*, 2007), ranging from one to less than 20 larvae (Anosike *et al.*, 2007;
422 Burkot *et al.*, 2007; Mangudo *et al.*, 2011). Even though average numbers of most species
423 collected were low, considerably higher average numbers of *Ae. aegypti* immature per tree hole
424 were detected in Orán (55 ± 13 , ranging from 1 to 733) (more details in Mangudo *et al.*, 2015),
425 highlighting the relevance of these larval habitats as dengue vector production sources. The
426 combined presence of *Ae. aegypti*, *Haemagogus* and *Sabethes* in the urban environment indicate
427 a potential risk of the study area of yellow fever and dengue epidemics and other arboviral
428 diseases.

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637 **Table 1.** Mosquito species collected (total number of specimens) in tree holes in urban and
 638 suburban environments of San Ramón de la Nueva Orán and adjacent forested areas, Salta
 639 province, Argentina.

Species	Environment			Total number (%)	Average	Positive samples (%) ^b
	Urban	Suburban	Forest		per tree hole (range) ^a	
					55.1 (1-	
<i>Ae. aegypti</i>	2038	3022	9	5069 (92.2)	733)	92 (54)
<i>Ae. terrens</i>	0	17	48	65 (1.2)	8.1 (1-23)	8 (5)
<i>Hg. leucocelaenus</i>	25	0	8	33 (0.6)	2.8 (1-9)	12 (7)
<i>Hg. spegazzinii</i>	91	21	27	139 (2.5)	3.9 (1-23)	36 (21)
<i>Sa. petrocchiaie</i>	0	5	18	23 (0.4)	2.9 (1-5)	8 (5)
<i>Sa. purpureus</i>	63	38	4	105 (1.9)	4.6 (1-33)	23 (14)
<i>Tx. guadeloupensis</i>	10	3	52	65 (1.2)	1.2 (1-3)	53 (31)
Estimated sample		0.98	0.99			
Coverage^c	1 (1)	(0.95)	(0.96)			
Total richness	5	6	7			
Effective number of						
species	1.47	1.17	5.22			

640 ^a**Average number per tree hole.**

641 ^b**Samples indicate total number of tree holes and sampling dates positive for Culicidae.**

642 ^c**Sample coverage C and rare species coverage in parenthesis.**

643 **Table 2.** Average mosquito species diversity (richness and effective number of species
644 (expression 1)) per tree hole in urban and suburban environments in the city of San Ramón de la
645 Nueva Orán and adjacent forested areas, Salta province, Argentina. Mean \pm standard errors are
646 shown.

	Environment		
	Urban	Suburban	Forest
Richness (S)	1.38 \pm 0.13	1.18 \pm 0.21	1.30 \pm 0.15
Effective number of species	1.43 \pm 0.23	1.22 \pm 0.12	1.31 \pm 0.16

647

648 **Table 3.** Mean abundances (ln(n+1)) per tree hole and percentage contribution to the
 649 dissimilarity between city (urban and suburban) and forest (according to SIMPER analysis) of
 650 mosquito species collected in tree holes, in the city of San Ramón de la Nueva Orán and adjacent
 651 forested areas, Salta province.

Species	City	Forest	Contribution
MD = 93,4%			
<i>Ae. aegypti</i>	2.26	0.04	49.07
<i>Tx. guadeloupensis</i>	0.07	0.56	17.07
<i>Hg. spegazzini</i>	0.30	0.22	10.18
<i>Sa. purpureus</i>	0.23	0.05	7.04
<i>Sa. petrocchiaie</i>	0.02	0.14	3.75
<i>Hg. leucocelaenus</i>	0.08	0.08	3.18
<i>Ae. terrens</i>	0.04	0.15	3.10

652 **MD = Mean dissimilarity**

653

654

Table 4. Number of tree holes where each species was found per habitat.

	Total tree				
	holes ^a	City		Forest	
		Single	W/other	Single	W/other
<i>Ae. aegypti</i>	29	16	12	0	1
<i>Ae. terrens</i>	3	0	1	0	2
<i>Hg. leucocelaenus</i>	8	1	4	0	3
<i>Hg. spegazzini</i>	15	1	7	2	5
<i>Sa. petrocchiaie</i>	7	0	1	2	4
<i>Sa. purpureus</i>	14	3	8	0	3
<i>Tx. guadeloupensis</i>	22	1	3	12	6

655

^a: Number of tree holes that were positive at least once for a given species.

656

Single: number of tree holes where the species was never found coexisting with another

657

species. W/other: Number of trees where the species was found at least once coexisting with

658

other (one or more) species.

659

660 **Table 5.** Number of samples where two species were collected together. Number in parenthesis
 661 indicates number of tree holes those samples came from.

	Habitat	<i>Ae. aeg</i>	<i>Ae. ter</i>	<i>Hg. leu</i>	<i>Hg. spe</i>	<i>Sa. pet</i>	<i>Sa. pur</i>	<i>Tx. gua</i>
<i>Ae. aeg</i>	City	64 (28)	3 (1)	5 (5)	18 (7)	0	10 (7)	3 (2)
	Forest	0 (1)	1 (1)	0	0	0	0	0
<i>Ae. ter</i>	City		0	0	2 (1)	0	0	0
	Forest		2 (2)	1 (1)	1 (1)	0	0	2 (2)
<i>Hg. leu</i>	City			1 (1)	5 (4)	0	2 (2)	0
	Forest			2 (1)	4 (3)	1 (1)	0	2 (1)
<i>Hg. spe</i>	City				4 (3)	0	3 (2)	1 (1)
	Forest				5 (4)	1 (1)	1 (1)	5 (3)
<i>Sa. pet</i>	City					0	1 (1)	1 (1)
	Forest					2 (1)	2 (2)	2 (2)
<i>Sa. pur</i>	City						6 (4)	2 (1)
	Forest						0	1 (1)
<i>Tx. gua</i>	City							5 (4)
	Forest							34 (16)

662 **In bold single species occurrences**

663 *Ae. aeg*: *Aedes aegypti*; *Ae. ter*: *Aedes terreus*; *Hg. leu*: *Haemagogus leucocelaenus*; *Hg. spe*:
 664 *Haemagogus spegazzini*; *Sa. pet*: *Sabethes petrocchiai*; *Sa. pur*: *Sabethes purpureus*; *Tx. gua*:
 665 *Toxorynchites guadaloupensis*.

666 **Figure Legend**

667 Figure 1. Location of study area and tree holes that harboured culicid larvae and/or pupae at least
668 once, on sidewalks and parks in urban areas (black figures) in the city of San Ramón de la Nueva
669 Orán and within a square area (50x50 m²) at three Yungas forest sites (white figures) located at
670 0.5 km to the northeast, 7 km to the east and 4 km to the southeast from the urban border, Salta
671 province, Argentina.

672

673 Figure 2. Species accumulation curves obtained using sample rarefaction and extrapolations
674 (continued lines) from the total number of samples collected on each habitat (urban in black and
675 square, suburban in dark grey and circle and forest in light grey and triangle) in San Ramón de la
676 Nueva Orán, Salta province, from a multinomial model, with 95% unconditional confidence
677 intervals (CI95%) (dotted lines) (based on Colwell *et al.*, 2012).

678

679

Figure 1

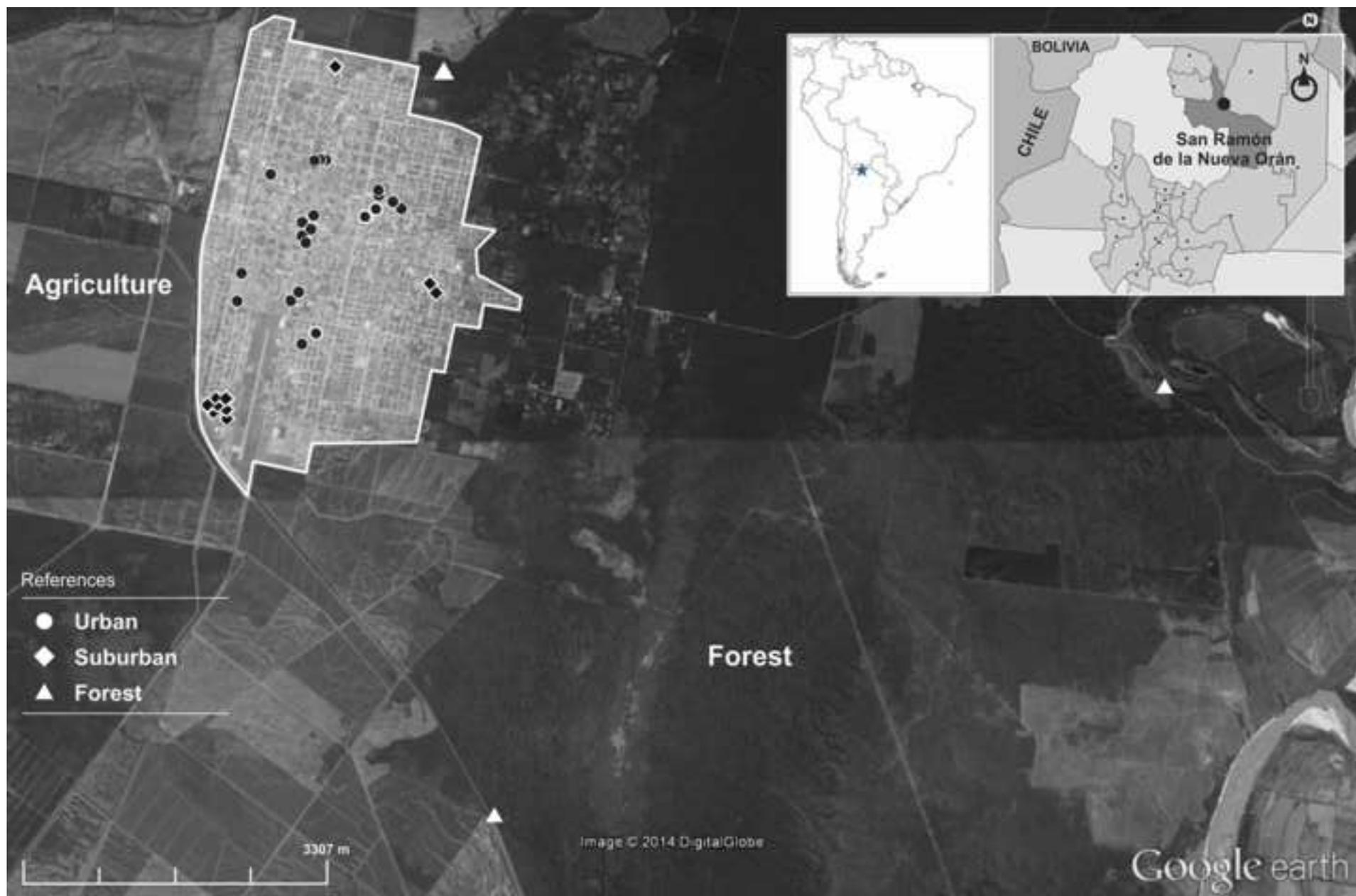
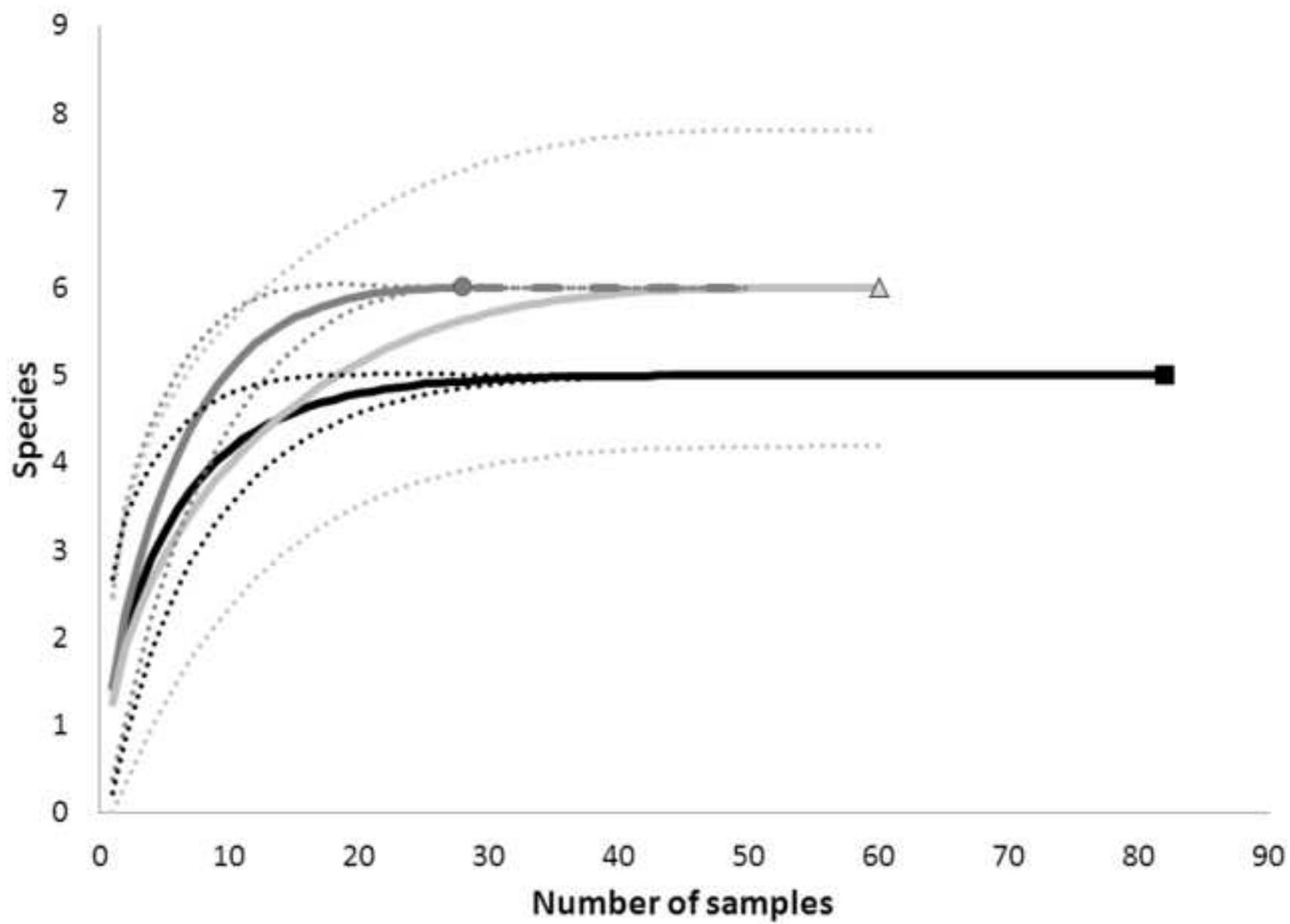


Figure 2





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Decision for BER-D-17-00068R1

2 mensajes

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30 de junio de 2017, 6:51

Responder a: BER Journal Office <entomology@cambridge.org>

Para: Raquel M Gleiser <rgleiser@crean.agro.uncor.edu>

CC: emilie.gray@coloradocollege.edu, cmangudo@hotmail.com, juan.p.aparicio@gmail.com, gustavo.c.rossi@gmail.com

Dear Dr. Gleiser,

Manuscript number: BER-D-17-00068R1

Title: Tree hole mosquito species composition and relative abundances differ between urban and adjacent forest habitats in northwestern Argentina

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On behalf of the Editorial Board

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RAQUEL MIRANDA GLEISER <raquel.gleiser@unc.edu.ar>

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Dear Valerie Dennis

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Best regards,

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