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Authors: M.V. Cardo, A. Rubio, M.T. Junges, D. Vezzani, A.E. Carbajo

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Heterogeneous distribution of *Culex pipiens*, *Culex quinquefasciatus* and their hybrids along the urbanisation gradient

Cardo MV[1,2], Rubio A[1,2], Junges MT[1,2], Vezzani D[2,3], Carbajo AE[1,2]

[1] Universidad Nacional de San Martín, Laboratorio de Ecología de Enfermedades Transmitidas por Vectores (2eTV), 3iA, General San Martín, Buenos Aires, Argentina

[2] Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

[3] Universidad Nacional del Centro de la Provincia de Buenos Aires, Facultad de Ciencias Exactas, Instituto Multidisciplinario sobre Ecosistemas y Desarrollo Sustentable, Tandil, Buenos Aires, Argentina

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ABSTRACT

The mosquitoes of the *Culex pipiens* complex, hereafter referred to as the Pipiens Assemblage, are vectors of arbovirus of worldwide concern including West Nile and St. Louis encephalitis. Given their distinct eco-physiology and vectorial capacity, accurate specimen identification and insight in the environmental drivers of their distribution are essential for the understanding of disease transmission patterns. Using a PCR-based identification protocol, we characterized the spatial distribution of *Cx. pipiens*, *Cx. quinquefasciatus* and their hybrids developing in used tyres located within the overlapping region in South America as a function of different estimators of the urbanisation gradient. Out of 84 samples collected from tyre piles of 20 sites, we identified 369 larvae which corresponded predominantly to *Cx. quinquefasciatus* (76.4% of immatures) all along the gradient but more frequent at the urban end. *Cx. pipiens* (21.4%) was more conspicuous at the low urbanised end but was also present in highly urbanised sites, whereas hybrids were collected in very low numbers (2.2%). The urbanisation estimator best associated with the heterogeneous occurrence of the Pipiens Assemblage members was the proportion of impervious surface 1km around each tyre pile, which explained 41.7% of the variability in the data, followed closely by the distance to the Capital City (38.3%). Cumulative annual precipitation, population number in a 1km radius around each pile and distance to the de la Plata River were significantly associated with the distribution of the Pipiens Assemblage at lower explanation percentages (20-23%). A thorough understanding of the ecological basis and environmental associations of the distribution of Pipiens Assemblage members will enable forecasting population trends in changing environments to develop effective control measures for mosquitoes and the diseases they transmit.

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Keywords

Pipiens Assemblage; arbovirus; polymerase chain reaction; demography; impervious area; discarded tyres.

INTRODUCTION

The mosquitoes grouped in the complex *Culex pipiens* s.l. Linnaeus, 1758 (Diptera: Culicidae), hereafter referred to as the Pipiens Assemblage to avoid difficulties associated with the taxonomic meaning of the word "complex" (Harbach 2012), are a focus of global ecological research due to their medical and veterinary relevance (Farajollahi *et al.* 2011). They transmit a wide variety of pathogens, such as worms that cause lymphatic filariasis (Farajollahi *et al.* 2011) and dirofilaria (Vezzani *et al.* 2011), and numerous viruses, including the ones that cause West Nile and St. Louis encephalitis (Solomon & Mallewa 2001). Both viral diseases are primarily enzootic among birds, with humans and equines serving as incidental hosts. It is estimated that 2.6 to 6.1 million people have been infected with West Nile virus in the United States since its introduction in 1999 (Chancey *et al.* 2015), with an economic burden of USD 778 millions (Barret 2014).

Each member of the Pipiens Assemblage presents distinct eco-physiological traits that affect its role as vector, in terms of seasonality, habitat selection, feeding preferences, diapause and autogeny (Vinogradova 2003). Also, members have been

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documented to present different vectorial capacity (Ciota *et al.* 2013, Cornel *et al.* 2003). Therefore, identifying the environmental drivers of their distribution is an essential milestone in the comprehension of arbovirus transmission cycles, to aid in their prevention and control. For this, a first unavoidable step is the accurate identification of individuals. The traditionally used DV/D ratio of the male phallosome (Sundararaman 1949) obviously cannot be performed on other life stages and is presumed to be affected by temperature, providing non reliable identifications (Wilton & Jakob 1985). A range of molecular assays have been recently developed for the same purpose (reviewed in Farajollahi *et al.* 2011).

In Argentina, the global southernmost extreme of its distribution, the Pipiens Assemblage is composed by *Cx. pipiens* (with two bioforms, *pipiens* and *molestus*) and *Culex quinquefasciatus* Say, distributed along temperate and subtropical areas, respectively. In the centre of the country, both members converge and hybridize according to morphometric studies (Almirón *et al.* 1995, Cardo *et al*. 2016, Diez *et al.* 2012). Only two identifications using molecular techniques have been conducted so far in the region. The first was a group of mosquitoes collected in the La Plata City and used for experimental studies of vector competence for West Nile virus in the US, which were identified as *Cx. quinquefasciatus* and *Cx. pipiens* form *molestus* (Micieli *et al.* 2013). The second was part of a broad study on the genus *Culex* through barcoding, which included four specimens of the Pipiens Assemblage (Laurito *et al.* 2013).

Urbanisation is associated with a profound alteration of the environment as a consequence of degradation, fragmentation and replacement of original natural habitats which, among other consequences, significantly increase soil impermeability through construction for transportation and buildings (Slonecker et al. 2001, Wessolek

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2008). Impervious surfaces are defined as any anthropogenic materials that water cannot infiltrate, mainly pavements covered by asphalt, concrete, brick or stone (e.g. roads, sidewalks, parking lots, airports), and rooftops. The proportion of impervious area has been recognized as an accurate predictor for urbanisation (Powell *et al.* 2008) and successfully associated with different attributes of Dipteran communities (e.g. Rubio *et al.* 2012, 2013). Besides, urban systems are an intricate mosaic of land uses with different levels of structural complexity and heterogeneous spatial disposition, in comparison with more homogeneous natural systems (Andersson 2006). Urbanisation may affect the spatial distribution pattern of mosquito species by offering different resource availability or degrees of continuity among optimal habitat patches (Carbajo *et al.* 2006). For the members of the Pipiens Assemblage, as for many other species, the urbanisation level could reflect vegetation structure, availability of different feeding sources and larval habitat types (Ferraguti *et al.* 2016).

The immatures of the Pipiens Assemblage utilize a wide variety of aquatic habitats for their development, a non-exhaustive list including ground habitats (stream margins, pond edges, temporary and permanent pools), natural (tree holes) and artificial containers (flower vases, buckets) (Vinogradova 2000). Among the latter, vehicle tyres are unavoidable components of domestic environments, from highly urbanised areas to agricultural lands. They are built with non-degradable materials and, when improperly discarded and filled with rainwater, they become a suitable habitat for immatures of mosquitoes (Yee 2008). The members of the Pipiens Assemblage have been recorded in these artificial habitats in high abundance (Rubio *et al.* 2011).

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Hybridisation between *Cx. pipiens* and *Cx. quinquefasciatus* occurs only in the Americas at temperate latitudes (Farahollaji *et al.* 2011). In North America, urbanisation has been associated with the spatial distribution of the members of the Pipiens Assemblage (e.g. Díaz-Badillo *et al.* 2011, Savage *et al.* 2006). Within the southern distribution overlapping region, we recently assessed the response of the members of the Pipiens Assemblage developing in flower vases from cemeteries to latitude and urbanisation gradients (Cardo *et al.* 2016). Whereas the former accounted for most of the spatial heterogeneity, the proportion of impervious area around each cemetery was associated with the occurrence of both *Cx. quinquefasciatus* and hybrids in positive and negative ways, respectively. No clear trend for *Cx. pipiens* was observed and, to our knowledge, no other studies have been conducted so far in the region. To gain further insight in the ecological determinants of the distribution of the members of the Pipiens Assemblage and its link to urbanisation, the aims of this study were (1) to perform PCR-based identifications of the members of the Pipiens Assemblage in Buenos Aires, within the overlapping region in South America; (2) to investigate the spatial distribution of *Cx. pipiens*, *Cx. quinquefasciatus* and their hybrids in relation to the urbanisation gradient within a fixed latitude; and (3) to select the optimal urbanisation estimator for this association.

MATERIALS AND METHODS

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Study area

The study area is a rectangle of approximately 0.27º latitude x 0.55º longitude (1,830 km^2, 34.74 - 35.01ºS, 58.67-58.12ºW) within Buenos Aires Province, Argentina. It is immersed in the suburbs of Buenos Aires City, the second megalopolis in South America and the capital of Argentina, which is located at the shore of the de la Plata River estuary (Fig. 1). The climate is temperate humid-subhumid, with an average temperature of 16.3ºC and cumulative annual precipitation of 946-992mm east-west, respectively. The original grassland has been dissected by communication roads and heavily influenced by human settlements. The area includes 7 districts which vary widely in population density (91 - 9,964 hab/km^2) (INDEC 2010) but are located at similar latitudes (maximum N-S distance approx. 30km, Fig. 1).

Sampling design

Information regarding the location of sites with tyre piles from Rubio *et al.* (2011) was updated by inspecting all main and secondary paved roads within the study area, in the search for outdoor piles. Five main arteries connecting Buenos Aires City with the surrounding suburbs were defined as natural transects (numbered T1 to T5 from west to east, Fig. 1). Along these, a total of 110 sites were geo-referenced, then reduced to 50 closest in space to minimize latitudinal and other unmeasured variability sources. The proportion of impervious area (PIA) in a circle of 1000m radius around each site was calculated (see details in Data Analysis section) and used to categorize the 50 selected sites in three urbanisation levels by natural breaks and Google Earth visual checking: low PIA≤0.46, middle 0.46<PIA<0.74, high PIA≥0.74 (Fig. 1). This

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categorization guaranteed a similar sampling effort throughout the urbanisation gradient.

Mosquito surveys were conducted bimonthly from December 2015 through April 2016, maintaining a similar sampling effort for the three campaigns (approximately 80 man hours by the same two operators). This period includes the maximum abundances of immatures and adults of the Pipiens Assemblage in the region (Vezzani & Albicócco 2009, Vezzani *et al.* 2011).

Mosquito collection

At each site, *Culex* mosquito larvae were collected of up to 6 water-filled tyres after homogenisation of the content using a fine-mesh strainer or a 300mL dipper. Immatures were first placed in a 30x15x5cm white tray with clean tap water. Then, 6 larvae of *Culex* sp. were placed individually in sterile 1.5mL eppendorfs containing physiologic solution for washing impurities and detritus. A pool of 10 larvae was collected in a 10mL falcon tube and treated likewise. If all samples could not be obtained in a site, the next one of the same urbanisation category was inspected until the 6 samples were achieved. Once in the laboratory, all specimens were fixed in 70% ethanol and preserved at –20ºC. Their correspondence to the Pipiens Assemblage was checked under stereoscopic microscope using a standard dichotomic key (Rossi *et al.* 2002) prior to DNA extraction.

Molecular assays

DNA isolation. Mosquito larvae were ground with mortar and pestle and genomic DNA was extracted using the Accuprep Genomic DNA Extraction kit (Bioneer, Korea),

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following the recommendations of the manufacturer for mammalian tissue. DNA was eluted in 100µl of elution buffer and stored at −20°C.

PCR analysis. The members of the Pipiens Assemblage were identified by means of a polymorphism in the second intron of the Ace-2 nuclear gen, following the PCR protocol set up by Smith & Fonseca (2004). Briefly, amplifications were carried out mixing 5X Green GoTaq® Reaction Buffer (Promega) with 250µM of each dNTP, 0.4µM of forward primer for *Cx. quinquefasciatus*, 0.2µM of forward primer for *Cx. pipiens*, 0.4µM of reverse primer and 1 unit of GoTaq® DNA polymerase (Promega). 4.8µl of autoclaved distilled water and 5µl of each DNA sample were added to make up a final reaction volume of 25µl. Thermal cycle reactions were set to a first denaturing step of 94°C for 5 min, followed by 35 cycles each composed of 94°C for 30 s, 55°C for 30 s, 72°C for 60 s, and a final step of 72°C for 5 min.

Gel electrophoresis. A 5µl aliquot of each amplified product was electrophoresed in 1.5% agarose gel containing ethidium bromide (0.5µg/mL) and 0.5X TBE buffer. Bands were visualised under a gel UV revealing system (Maestrogen). PCR products produce a 610 base pair (bp) fragment for *Cx. pipiens*, 274 bp for *Cx. quinquefasciatus* and both fragments for hybrids. As the optimal amount of starting material for DNA isolation was 25mg (equivalent approximately to 5 larvae), to try to represent each container more thoroughly analyses were first performed with mosquito pools consisting of 10 halves larvae or 4-5 larvae when 10 larvae were not available. Only if the two bands were obtained, we proceeded to extract, amplify and run the individual samples. A 1 kb DNA Ladder (PB-L, Quilmes, Argentina) was run in parallel to allow size estimation of observed bands. Larval samples from Quitilipi (26.87ºS, 60.22ºW) and Necochea (38.55ºS, 58.73ºW) were used as positive controls for *Cx. quinquefasciatus* and *Cx.*

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pipiens, respectively. Both collection sites are > 250km distant from the hybridisation region proposed by Diez *et al.* (2012) (Fig. 1).

Data Analysis

Two direct estimators of urbanisation were calculated. (A) PIA: a Landsat 5 TM satellite image (30m × 30m resolution) captured in January 22, 2010 was used to perform a non-supervised classification to identify impervious areas in a circle of 1km radius around each site using Erdas Imagine 8.4 and GIS-ArcView 3.2 softwares. (B) Pop1k: population number in a circle of 1km radius around each site was calculated based on demographic data from the last national census at census tract level (INDEC 2010) and spatial processing.

Mean annual temperature (Tmean) and cumulative annual precipitation (Ppcum) were also evaluated (average between 2005 and 2010 for both climatic variables, SADS 2014). Finally, the distances to Buenos Aires City nearest border (dist_city) and to the de la Plata River (dist_river) were calculated for each site.

Pearson´s R-squared correlation coefficient between each pair of explanatory variables was calculated. Given the high level of correlation expected among different urbanisation estimators, Principal Component Analysis (Jolliffe 2002) was performed to extract the first orthogonal components and use them as non-correlated input variables in multivariate modelling. Generalized Linear Mixed Models were used to analyse the association between the spatial distribution of the members of the Pipiens Assemblage and the six continuous explanatory variables (PIA, Pop1k, Tmean, Ppcum, dist_city and dist_river), along with the first three principal component axes (PC). The response variable was defined as an Assemblage Index which took different values

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according to the occurrence of one or more members per site: -1 for *Cx. pipiens* alone, 0 for hybrids alone, 1 for *Cx. quinquefasciatus* alone and the average of these values for members joint occurrence (e.g. 0.5 if *Cx. quinquefasciatus* and hybrids coexist in a site, and 0 if the three members are found together). We followed a stepwise forward procedure, in which explanatory variables were entered one-by-one, along with squared relations and two-way interactions without colinearity issues. These were tested in every step using the variance inflation factors (VIF), requiring all values to be < 5 (Zuur *et al.* 2009). Errors were normally distributed (link identity) and the clusters defined by the sampling design (month and transect) were entered as random grouping factors. Analyses were performed using the open-source software R 3.2.3, with lme4, car and boot packages (R Core Team 2015).

RESULTS

As expected, pairwise correlations between almost all the explanatory variables were high (Table 1). A larger distance to the city border was associated with less impervious area, lower mean temperature, precipitation and population density. The proportion of impervious area was positively associated with the mean temperature. Both direct estimators of urbanisation, the population number in 1km radius and the proportion of impervious area, were also highly associated. Distance to the river was mainly correlated with the precipitation gradient, wetter in the east and drier in the west.

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The first three components of the Principal Component Analysis explained 97% of the variability in the data (75, 16 and 6% for axis 1, 2 and 3, respectively). PC1 was negatively associated with the proportion of impervious area, mean temperature and population density, and positively with distance to the river and the city, whereas PC2 was positively associated with distance to the river and population density and moderately and negatively related to cumulative precipitation (Table 2, Fig. 2).

PC1 segregated sites mainly as a function of the proportion of impervious area, therefore accurately grouped those corresponding to high, mid and low urbanisation levels (Fig. 2). Variability along PC2 was higher for the high category, given its variable location regarding the river and more heterogeneous population density.

Culex larvae were found and collected in 26 out of the 50 pre-selected sites. In transects 1, 2 and 3, the three members of the Pipiens Assemblage were identified, whereas in transects 4 and 5, only *Cx. quinquefasciatus* was recorded (Fig. 1). In the latter two, the subset of sites with larvae that could be successfully identified (3 sites in each transect) were only located in suburban areas. Therefore, transects 4 and 5 were omitted from further analyses, as the urbanisation gradient was not adequately represented and its inclusion would have given more weight to the middle portion of the gradient.

Within the selected subset, high, middle and low urbanisation categories were represented by 7, 7 and 6 sites, respectively. We obtained samples from 84 tyres, with a high bias towards the urban end of the gradient (44, 27 and 13 samples for high,

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middle and low urbanisation, respectively). All urbanisation categories were present in the three sampling events (Table 3).

PCR-based identification of the members of the Pipiens Assemblage was successful in 56% of the samples (47/84, the remaining were either degraded or not correctly processed). A total of 369 immatures were identified, 282 of which corresponded to *Cx. quinquefasciatus*, 79 to *Cx. pipiens* and 8 to hybrids. *Cx. quinquefasciatus* and *Cx. pipiens* were collected alone in 10 and 3 sites, towards the high and low ends of the urbanisation gradient, respectively (Fig. 3). At intermediate values of impervious area, they were found coexisting in 3 sites, along with the pair *Cx. quinquefasciatus* - hybrids in 4 sites (Fig. 3). No collection of hybrids alone was obtained.

According to univariate analyses, the proportion of impervious area was best associated with the Assemblage Index was and explained 41.7% of the total variance, followed closely by the distance to the city and the first axis of the Principal Component Analysis (Table 3). Positive values of the Assemblage Index (indicative of *Cx. quinquefasciatus* and, to a lesser extent, hybrids) were associated with higher imperviousness, whereas the opposite occurred with the distance to the city. This was expectable given that both explanatory variables were highly and negatively correlated (R^2 = -0.89, Table 1).

Population density, distance to the river and cumulative precipitation were also associated with the Assemblage Index and explained around 20-23% of the variance (Table 3). Positive values of the Assemblage Index were associated with sites closer to the river, with more precipitation and higher population density. Only the first

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component of the Principal Component Analysis, which presented a high load of the percentage of impervious area (Table 2), was significantly associated with the Assemblage Index (Table 4).

After the addition of any first variable, no second variable (including squared relations and two-way interactions) were significant. Random grouping factors were also not significant.

DISCUSSION

According to our findings, the composition of the Pipiens Assemblage developing in vehicle tyres within Buenos Aires is heterogeneous along the urbanisation gradient. *Cx. quinquefasciatus* is presumably the most widespread and conspicuous member of the Pipiens Assemblage utilizing tyre piles, recorded practically all along the gradient although more frequently at the urban end. On the contrary, *Cx. pipiens* was more frequent at the low urbanised end but was also present in highly urbanised sites. A similar pattern was reported in a survey of cemetery flower vases in Mexico City, within the North American overlapping region (Díaz-Badillo *et al.* 2011). There, *Cx. pipiens* was detected mainly in suburban or rural lands and *Cx. quinquefasciatus* was collected primarily in urban zones.

Among a set of variables depicting urbanisation, we identified the proportion of impervious area as best associated with the spatial distribution of the Pipiens Assemblage developing in tyres, followed by the distance to the de la Plata River. The latter variable is easy to obtain and may be a proxy for the precipitation gradient.

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However, the particular situation of Buenos Aires City and suburbs located contiguous to a major river estuary certainly does not hold for other cities with different spatial configuration and geography. In turn, imperviousness is a direct measure of urbanisation in the surroundings of the sampling sites, calculated by an easily comparable standardized procedure (McDonnell & Hahs 2008). It was also a predictor of the spatial distribution of the members of the Pipiens Assemblage utilizing cemetery flower vases at a broader scale, a 100km latitudinal transect in Buenos Aires (Cardo *et al.* 2016). Although highly correlated with the proportion of impervious area, population density was less associated with the distribution of the Pipiens Assemblage. This could be due to the occurrence of highly impervious land uses with low population, such as industries.

Urbanisation has profound effects on both vertebrate and invertebrate communities, often leading to an increase in the abundance of a few species with a concomitant loss of biodiversity (McKinney 2002). The increased dominance of key species of both vectors and hosts could favour their interspecific contact rates and increase pathogen transmission rates (Woolhouse *et al.* 2001). According to their feeding behaviour, some vector species may facilitate the transmission between reservoir vertebrate hosts, while others may act as bridges between infected competent vertebrates and humans or other susceptible species (Ferraguti *et al.* 2016). Each member of the Pipiens Assemblage presents a distinct feeding behaviour. *Cx. pipiens* form *pipiens* is mainly ornitophilic, but also feeds on mammals including humans, whereas *Cx. pipiens* form *molestus* is highly anthropophilic. *Cx. quinquefasciatus* preferences vary according to the geographical location from 100% mammalophilic, with many meals taken on humans, to a high degree of ornithophily

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(Takken & Verhulst 2013, Vinogradova 2003). Hybrids are considered of great epidemiological importance as they may display a more opportunistic biting behaviour (Hamer *et al.* 2008). The study of the feeding preferences of each member in the study area is pending.

We recorded considerably less coexistence of *Cx. pipiens*, *Cx. quinquefasciatus* and hybrids per container and site in used tyres than in cemetery flower vases of the study area (Cardo *et al.* 2016). However, as the previous study identified the individuals based on male genitalia morphometrics, it is uncertain whether the differential results are due to a distinct behaviour of the Pipiens Assemblage according to the container type or to discrepancies between identification techniques. Although some authors have found concomitant results between the morphological and the PCR-based approach (de Morais *et al.* 2010, Diaz-Badillo *et al.* 2011), others have reported incongruences (Cornel *et al.* 2003, Gao *et al.* 2016, McAbee *et al.* 2008). It has been argued that the DV/D ratio, although useful to identify *Cx. quinquefasciatus* and *Cx. pipiens* in pure populations, does not accurately separate them in areas where there has been extensive introgression (Sanogo *et al.* 2008), which would be the case for the region under study.

We did not differentiate between both *pipiens* forms, for which differential distribution between urban and natural areas have been recently reported in Europe (Martinez de la Puente *et al.* 2016). So far, only *Cx. pipiens* form *molestus* has been identified in the study area (Micieli *et al.* 2013). If *Cx. pipiens* form *pipiens* were also present, and given their eco-physiological differences relevant to vector transmission, the spatial distribution of each form should be evaluated in further studies. Also, microenvironmental factors known to be associated with the occurrence of culicids in

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containers, such as vegetation cover and shade (Vezzani 2007, Yee 2008), were out of the scope of this work. It has been recorded that the Pipiens Assemblage is more frequent in vegetated and shaded habitats (Rubio *et al.* 2011, Vezzani & Albicócco 2009). A differential effect of these factors on each member remains an open question.

Our results show for the first time that *Cx. pipiens*, *Cx. quinquefasciatus* and their hybrids are present in the Buenos Aires metropolitan area, and heterogeneously distributed along the urbanisation gradient when developing in used tyres. As differential oviposition preference or immature survival per habitat type may be operating, further studies focusing on other larval habitats and the adult population are needed to generalise the link between the urbanisation gradient and the distribution of the Pipiens Assemblage presented herein. Notwithstanding, we provide preliminary evidence that competent vectors for West Nile and St. Louis encephalitis viruses occur in a region in which the great abundance of migratory birds alert on the risk of entry of these and potentially other arboviruses. A human outbreak of St. Louis encephalitis was registered in Córdoba City, Argentina during 2005, with 47 people infected and 9 diseased (Díaz *et al.* 2006). The first epidemic outbreak in Buenos Aires City occurred in 2010, with 13 reported cases (Seijo *et al.* 2011). Regarding West Nile, although no human cases have been reported, the virus has been isolated from horses and birds (Diaz *et al.* 2008, Morales *et al.* 2006, Tauro *et al.* 2012). As it has been suggested that hybridisation between *Cx. pipiens* and *Cx. quinquefasciatus* mosquitoes may have facilitated the rapid spread of the disease in North America (Fonseca *et al.* 2004), increased surveillance and effective vector control should be implemented in order to avoid the risk of epidemics in this highly populated area.

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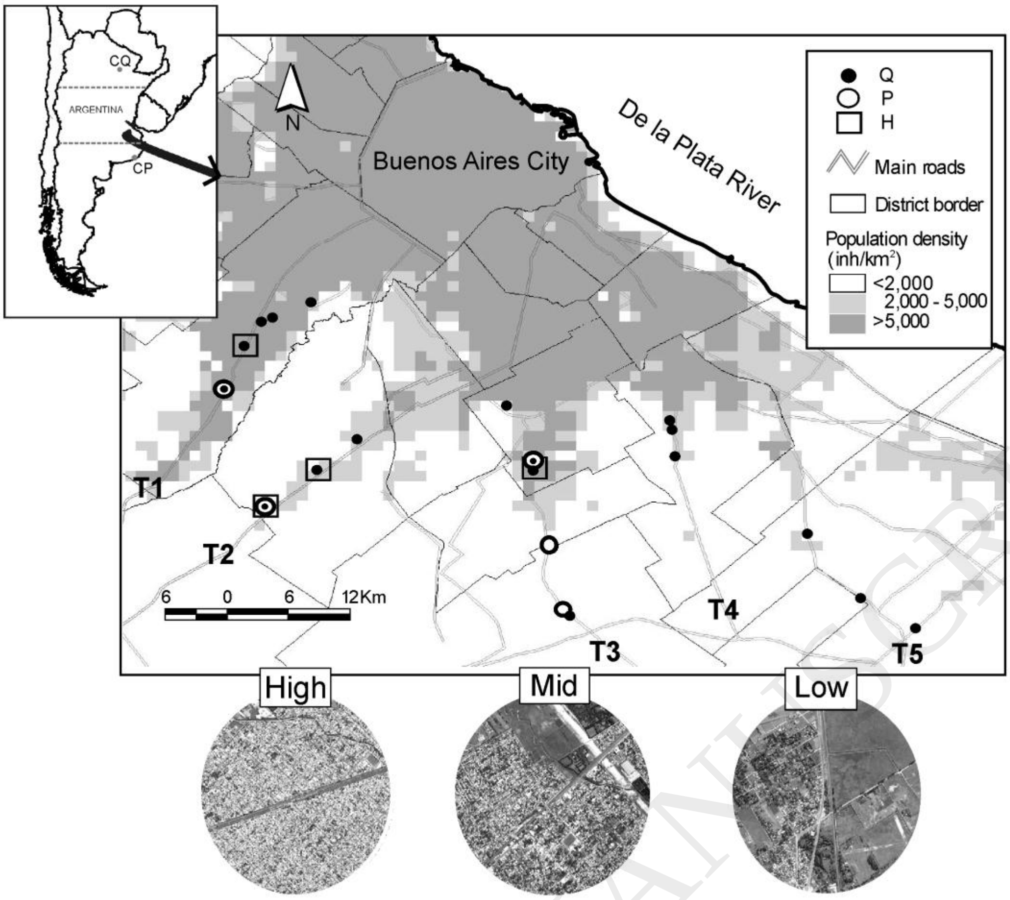

Fig. 1. Location of tyre piles in which members of the Pipiens Assemblage were collected and identified (filled circle for *Culex quinquefasciatus* -Q-, empty circle for *Cx. pipiens* -P- and empty square for their hybrids -H-) along five transects (labelled T1-T5 from west to east). A human population density map (Cardo *et al.* 2014) is superimposed for visualization purposes, with three categories (<2,000, 2,000-5,000 and >5,000 inhabitants/km^2) that approach urbanisation categories (0.88 correlation). Bottom circles exemplify the three urbanisation categories defined by satellite imagery. Upper left: position of the study area in southern South America. Dotted grey lines indicate the limits of the distribution of hybrids according to morphometric studies following Diez *et al.* (2012). Collection sites of the positive controls for *Cx. pipiens* and *Cx. quinquefasciatus* are denoted CP and CQ, respectively.

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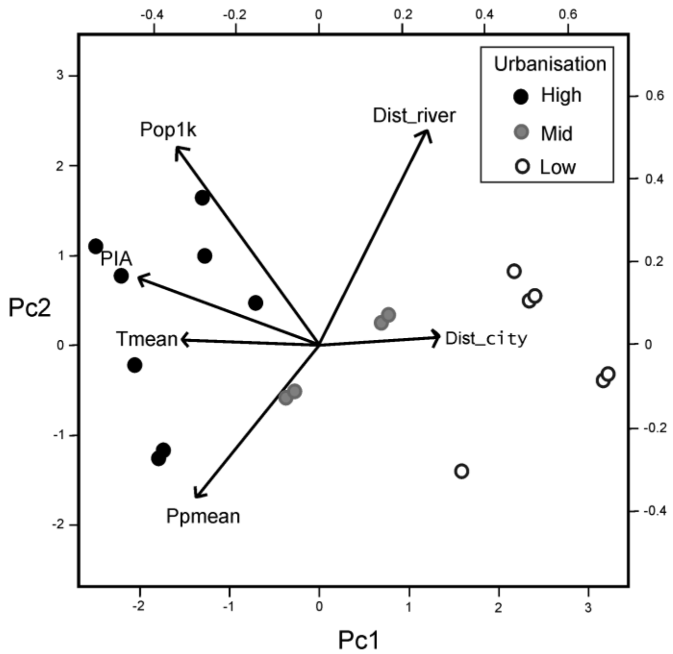

Fig. 2. Biplot of the Principal Component Analysis showing the contribution of explanatory variables to the first two Principal Components (PC), and sites ordered in the bidimentional space according to urbanisation categories.

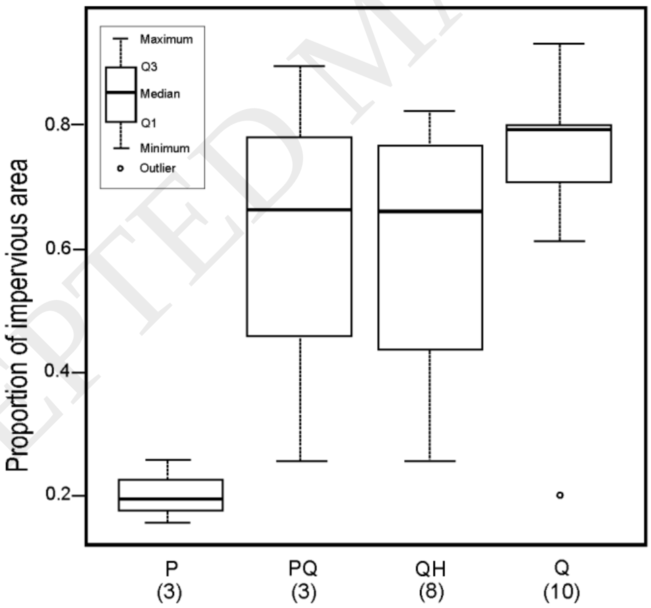

Fig. 3. Median and extreme values of the proportion of impervious area 1000m around each tyre pile for each of the combinations of members of the Pipiens Assemblage collected per site. P, Q and H represent *Cx. pipiens*, *Cx. quinquefasciatus* and hybrids, respectively. Numbers

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between brackets at the x-axis indicate the number of tyre piles in which each combination took place.

Table 1. Pearson´s R-squared pairwise correlation between explanatory variables. Asterisks next to the values indicate statistical significance. *p<0.05, **p<0.01, ***p<0.001.

	Dist_river	Tmean	Ppcum	PIA	Pop1k
Dist_city	0.67**	-0.88***	-0.84***	-0.89***	-0.76***
Dist_river		-0.50*	-0.90***	-0.59**	-0.28
Tmean			0.74***	0.76***	0.65**
Ppcum				-0.70***	0.46*
PIA					0.84***

Table 2. Variable loadings (i.e., eigenvectors) for each of the first three Principal Components (PC) extracted from the analysis performed on explanatory variables.

	PC1	PC2	PC3
PIA	-0.53	0.20	-0.31
Dist_river	0.32	0.64	0.38
Dist_city	0.36	0.02	-0.15
Ppcum	-0.36	-0.45	0.05
Tmean	-0.41	0.02	0.81
Pop1k	-0.43	0.60	-0.29

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Table 3. Number of tyre piles and tyres (denoted piles_tyres) in which immatures of the Pipiens Assemblage were collected, for each sampling campaign and urbanisation category.

* The same tyre pile was visited in two occasions.

	Urbanisation category		
	High	Mid	Low
December 2015	2 _ 12	3 _ 12	2 _ 4
February 2016	2_ 8	2 _ 8	1 _ 3
April 2016	4 _ 24	3 _ 7	3 _ 6
Total	7*_44	7_27	6_13

Table 4. Univariate models for the occurrence of the members of the Pipiens Assemblage in tyre piles within the sympatric region in Buenos Aires (see text for explanatory variable definitions). Asterisks next to the values indicate statistical significance *p<0.05, **p<0.01, whereas no significant association is denoted n.s.

	Parameter sign	Residual variance	Explained (%)
Null model		39.8	
PIA	+	23.2	**41.7****
Dist_city	-	24.6	**38.3****
PC1	-	25.6	**35.7****
Ppcum	+	30.8	22.7*
Pop1k	+	31.4	21.0*
Dist_river	-	31.5	20.8*
Tmean		33.0	n.s.
PC3		38.2	n.s.
PC2		39.8	n.s.

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