

***Culex saltanensis* and *Culex interfor* (Diptera: Culicidae) are susceptible and competent to transmit St. Louis encephalitis virus (Flavivirus: Flaviviridae) in central Argentina**

Round #1

by Anna Cohuet, 2019-09-17

PCI entomology: revision requested

The reviewers highlighted the interest of the manuscript but also pointed out some limits that need to be considered/corrected before recommendation can be considered. I invite the authors to revise the manuscript accordingly and to re-submit.

Response: changed as suggested, thanks!

Reviews

Reviewed by anonymous reviewer, 2019-09-16

The results reported in this study are interesting and are a worthwhile contribution to the field of medical entomology. Three *Culex* mosquitoes (*Cx. saltanensis*, *Cx. interfor*, *Cx. p. quinquefasciatus*) were experimentally infected with a strain of St. Louis encephalitis virus; and the vectorial competence of these three species was characterized using 3 traits: (i) infection rate (presence/absence of virus in mosquito abdomen), (ii) dissemination rate (presence/absence of virus in mosquito legs), and (iii) transmission rate (presence/absence of virus in mosquito salivary glands). The results indicate that these three species are competent for the development of this strain of SLEV. In particular, the authors found that 8/12 abdomens of *Cx. saltanensis* were infected vs 14/25 in *Cx. interfor* and 13/39 in *Cx. p. quinquefasciatus*. The virus successfully disseminated to the mosquito legs with the following prevalence: 8/12 for *Cx. saltanensis*, 10/25 for *Cx. interfor* and 7/39 for *Cx. p. quinquefasciatus*. Finally, the virus invaded mosquito salivary glands at the following rates: 2/12, 5/25, and 7/39 in *Cx. saltanensis*, *Cx. interfor* and *Cx. p. quinquefasciatus*, respectively.

Response: We quantified viral particles of SLEV on the expectorated saliva the three *Culex* species studied as described by (Richards et al., 2012; Anderson et al., 2010; Ebel et al., 2005), but salivary glands were not dissected (line 145-147). Then, transmission rate was determined by the number of mosquitoes with positive saliva of the total number of mosquitoes that blood fed (Line 182-184). This rate assumes that viral particles available in the expectorated saliva are able to infect a host. Because, virus presence and load were analyzed by VERO cell infection we partially addressed transmission. We do not know how many viral particles are needed to produce infection in the host and that question is part of a future study.

Richards SL, Anderson SL, Lord CC *et al.* Relationships between infection, dissemination, and transmission of West Nile virus RNA in *Culex pipiens quinquefasciatus* (Diptera: Culicidae). *J Med Entomol* 2012; 49: 132–142.

Anderson SL, Richards SL & Smartt CT. A Simple method for determining arbovirus transmission in mosquitoes. *J Am Mosq Control Assoc* 2010; 26: 108–111.

Ebel GE, Rochlin I, Longacker J *et al.* *Culex restuans* (Diptera: Culicidae) Relative Abundance and Vector Competence for West Nile Virus. *J. Med. Entomol* 2005; 42: 838-843.

While previous studies already characterized the competence of *Cx. p. quinquefasciatus*, this is the first time that the intrinsic competence of *Cx. saltanensis* and *Cx. interfor* is examined. This work therefore provides proof of principle that *Cx. saltanensis* and *Cx. interfor* are permissive species for the development of SLEV. The authors also analyzed statistical difference in infection, dissemination and transmission rates among the

three mosquito species. Although it would have been interesting, such comparisons cannot be derived from the current dataset. Analyses examining such differences are misleading for two reasons. First, while the sample sizes used in this experiment are sufficient to provide proof of principles that *Cx. saltanensis* and *Cx. interfor* are permissive species, they are too low to draw robust interspecific differences. Second, and most importantly, each mosquito species received different infectious blood-meals in this experiment. Unless I misunderstood, lines 200-203 and the raw data (excel file) indicates that *Cx. saltanensis* mosquitoes were fed on an infected chick with a viremia of $3.2 \log_{10}$ PFU/ml, *Cx. interfor* mosquitoes were fed on another infected chick with a viremia of $3.5 \log_{10}$ PFU/ml, while *Cx. p. quinquefasciatus* mosquitoes were fed on an infected chick with a viremia = $2.9 \log_{10}$ PFU/ml). Because the three mosquito species were not fed on the same infected chicks during the experiments, no competence comparison among species can be made. I apologize if this is a misunderstanding and if mosquitoes from different species indeed fed on the same individual chicks. On a similar note, because mosquito species seems confounded with chick viremia (e.g. all mosquitoes from the species *Cx. saltanensis* fed on a chick with a viremia of $3.2 \log_{10}$ PFU/ml) one cannot account for viremia in the binomial model. Using the raw data provided by the authors and the model described in the statistic section (lines 184-196), the following model: GLM (Results~Specie+viremia, family=binomial) do not allow to derive any statistics for the effect of viremia (same is true when viremia is considered a categorical variable with three levels instead of a numeric variable).

Response: Viremia dose was included as a co-variable to account for the unequal distribution among species in the experiments. In fact, adding this co-variable in the model helped to answer the design constrain and uncertainty derived by working with live animals as source of viremia (individual host response). The size of the chick made it difficult to feed all *Culex* species simultaneously from the same viremic host. Also, as individual chick cannot be switched between feeding trails (kinetic of viremia and animal welfare). For instance, between the two feeding attempts in *Culex p. quinquefasciatus* the lower one ($2.9 \log_{10}$ PFU/ml) was almost half log below the lower feeding attempt in *Cx. interfor* ($3.5 \log_{10}$ PFU/ml) setting plausible scenarios of dose dependence in the outcomes (infection, dissemination and transmission). Insofar, we believe that this potential dependence structure must be accounted during analysis. Moreover, accounting for this source of variation (even when it was not significant) improve the performance of the model and the estimation of marginal effect (difference between species). Thus, the aim of the experiment was explored the variation in the rates of infection, dissemination and transmission between two potential *Culex* vectors to respect *Cx. p. quinquefasciatus*, while experimental sources of variation were considered. Viremic variations in this range shown not are related to significant variations in infection, dissemination and transmission rates both intra and inter species (Richards et al., 2009, Smith et al., 2005).

Line 200-204 “There was a narrow range of viremia during blood feeding (*Cx. p. quinquefasciatus*= $2.9 \log_{10}$ PFU/ml, *Cx. interfor*= $3.5 \log_{10}$ PFU/ml and *Cx. saltanensis*= $3.2 \log_{10}$ PFU/ml) not contribute to the variation in infection (LR $X^2=1.01$, $df=2$, $n=76$, $p=0.604$), dissemination (LR $X^2=4.94$, $df=2$, $n=76$, $p=0.084$) and transmission (LR $X^2=1.20$, $df=2$, $n=76$, $p=0.548$).” This paragraph clearly states that the null hypothesis were not rejected against the viremia effect as alternative hypothesis, therefore the probability of infection, dissemination and transmission were “independent” of the three levels of viremia assayed.

Generalized Linear Models (GLM) comprise a complete toolbox for dealing with several error family distributions; among them success of infection (or dissemination/transmission) is expected to follow a binomial distribution. Given the structure of our experimental design and background knowledge of SLEV transmission system GLM could improve outcome estimations. Differences between success-fail events are distributed in a binomial space (logit scale). The scope of our analysis was not exploratory because the three species have been ecologically (*Cx. saltanensis*, *Cx. interfor*, *Cx. p. quinquefasciatus* [3, 8, 9, 14] and experimentally *Cx. p. quinquefasciatus* [8] incriminated in SLEV transmission in Argentina, as was stated in the introduction section. Also, GLM easily deal with co-variables to account for other sources of variation potentially masking the magnitude of differences. Here, we accounted by the narrow, but unequal, viremia levels between feeding trails. Therefore, a model-based analysis was selected to challenge the hypothesis “SLEV is transmitted by *Cx. saltanensis* and *Cx. interfor* in the central area of Argentina”.

Richards SL, Lord CC, Pesko K *et al.* Environmental and Biological Factors Influencing *Culex pipiens quinquefasciatus* Say (Diptera: Culicidae) Vector Competence for Saint Louis Encephalitis Virus. *Am J Trop Med Hyg* 2009; 81: 264–272.

Smith DR, Carrara AS, Aguilar PV et al. Evaluation of methods to assess transmission potential of Venezuelan Equine Encephalitis virus by mosquitoes and estimation of mosquito saliva titers. *Am J Trop Med Hyg* 2005; 73: 33–39.

[3]Diaz LA, Flores FS, Quaglia A *et al.* Intertwined arbovirus transmission activity: Reassessing the transmission cycle paradigm. *Front Physiol* 2013. 3: 493.

[8]Diaz LA, Flores FS, Beranek M et al. Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Córdoba, Argentina. *Trans R Soc Trop Med Hyg* 2013; 107: 332-334.

[9]Diaz LA, Ré V, Almirón W *et al.* Genotype III Saint Louis encephalitis virus outbreak, Argentina, 2005. *Emerg Infect Dis* 2006; 128: 1752-1754.

[14]Batallán GP. Binomía de *Culex interfor* Dyar en Córdoba, Argentina. [theses]. Córdoba: Universidad Nacional de Córdoba; 2013. 197 p.

The whole discussion is developed around the idea that, because *Cx. saltanensis* and *Cx. interfor* are abundant and permissive to the dissemination of SLEV in their salivary glands, they are possible vectors and may transmit the disease. This could be true but one critical factor must be fulfilled: real contact rate between competent vertebrate hosts and these vectors. What is the trophic preference and blood-feeding pattern of these two species? In fact, *Cx. saltanensis* and *Cx. interfor* will have the potential to ensure transmission, provided that they can feed on competent vertebrate hosts in natural conditions. A paragraph about the blood-feeding behavior of these two species would be great.

Response: changed as suggested. We added “*Culex interfor* and *Cx. saltanensis* are mainly ornithophiles, and bloodmeals from Columbiformes and Passeriformes have been also detected, although the pattern of host preference and its drivers have not been established yet [14; 38; 39]. In Argentina, *Z. auriculata* and *C. picui* are amplifier hosts of SLEV and have been recorded in engorged *Cx. saltanensis*, *Cx. interfor* and *Cx. p. quinquefasciatus* sustaining that SLEV maintenance could be relied on multiple vectors [3; 36]”...

[3]Diaz LA, Flores FS, Quaglia A *et al.* Intertwined arbovirus transmission activity: Reassessing the transmission cycle paradigm. *Front Physiol* 2013; 3: 493.

[14]Batallán GP. Binomía de *Culex interfor* Dyar en Córdoba, Argentina. [theses]. Córdoba: Universidad Nacional de Córdoba; 2013. 197 p.

[36]Diaz LA, Flores FS, Quaglia IA *et al.* Evaluation of Argentinean birds species as amplifying hosts for St. Louis encephalitis virus (Flavivirus, Flaviviridae). *Am J Trop Med Hyg* 2018; 99: 216–221.

[38]Beranek MD. Mosquitos del género *Culex* (Diptera: Culicidae) como vectores del virus Saint Louis encephalitis (Flavivirus) en ambientes urbanos de la ciudad de Córdoba [theses]. Córdoba: Universidad Nacional de Córdoba; 2019. 188 p.

[39]Berrón CI. Preferencia de hospedadores aviares en especies de mosquitos Género *Culex* asociadas a la transmisión de Flavivirus (Flaviviridae) en el arco sur de la Laguna Mar Chiquita. [theses]. Córdoba: Universidad Nacional de Córdoba; 2014. 155 p.

If contacts do occur, then what would be the most likely scenario and their role in disease transmission? Would *Cx. saltanensis* and *Cx. interfor* mostly maintain transmission among non-human reservoirs? Could they act as a bridge vector between birds and human? Or could they even ensure robust human transmission? Finally, what was the mortality rate of mosquitoes from 1 to 14 dpi? The Mat & Meth section mentions this was recorded. It would be important to report any lifespan difference among mosquito species as this is a major trait of vectorial capacity.

Response: changed as suggested. We added “*Culex saltanensis* and *Cx. interfor* participate in the maintenance of SLEV and could assist in the spillover of SLEV to humans. In 2004, prior to the outbreak of encephalitis in Córdoba City, SLEV infected *Cx. interfor* were detected [11]. In 2010, there were small outbreaks of SLEV in provinces, e.g., Buenos Aires, Córdoba and San Juan [37]. Not long thereafter, SLEV infected *Cx. saltanensis* were detected for first time in Córdoba City [14]. *Culex interfor* and *Cx. saltanensis* are mainly ornithophiles and bloodmeal from Columbiformes and Passeriformes have been also detected, although the pattern of host preference and its drivers have not been established yet [14; 38; 39]. In Argentina, *Z. auriculata* and *C. picui* are amplifier hosts of SLEV and have been recorded in engorged *Cx. saltanensis*, *Cx. interfor* and *Cx. p. quinquefasciatus* sustaining that SLEV maintenance could rely on multiple vectors [3; 36]. However, the transmission load in SLEV episystem could be unequal between the three *Culex*, despite they showed similar transmission rate experimentally (ranged 17-20%). For instance, among other traits, lifespan difference among mosquito species is expected to impact vector capacity as longest lifespan increase the odds of extrinsic incubation completeness and delivering infectious bites [7; 25]. Here, *Cx. p. quinquefasciatus* was less able to survive after a viremic bloodmeal than *Cx. saltanensis* and *Cx. interfor* suggesting that the role of the last species has been neglected. In addition, it has been proposed that *Cx. interfor* could transmit SLEV from birds to mammals and thus fulfill a role of “bridge vector” [14; 38; 40] as *Cx. interfor* was recorded in human baited barley traps [40] and *Cx. interfor* and *Cx. saltanensis* switch between bird feeding profile in spring-summer to bird-mammals in autumn in a rural environment [41]. The local populations of *Culex* spp. increase in abundance with peaks in summer, with are temporal distribution of *Culex* spp. coinciding with the activity peaks of SLEV in human infection [3, 42]. Adult mosquitoes belonging to the species *Cx. saltanensis* and *Cx. interfor* have been found in increasing numbers in Córdoba City, with a higher abundance in urban and periurban areas where vegetation is more robust. This differs from *Cx. p. quinquefasciatus*, which is predominant throughout a vast range of city-type environments [42]. Our results support the hypothesis that SLEV is transmitted by multiple sympatric *Culex* spp., and that both *Cx. saltanensis* and *Cx. interfor* can be considered potential vectors of SLEV.

Minor comments:

20 infected chicks were obtained (lines 133) but only 3 were used. Lines 200-202 “There was a narrow range of viremia during blood feeding (*Cx. p. quinquefasciatus*=2.9 log₁₀ PFU/ml, *Cx. interfor*=3.5 log₁₀ PFU/ml and *Cx. saltanensis*=3.2 log₁₀ PFU/ml)? This is unclear.

Response: We did not use 20 infected chicks; only used 3 infected chicks, one for each *Culex* species studied. “There was a narrow range of viremia during blood feeding (*Cx. p. quinquefasciatus*=2.9 log₁₀ PFU/ml, *Cx. interfor*=3.5 log₁₀ PFU/ml and *Cx. saltanensis*=3.2 log₁₀ PFU/ml”). We added in line 133 “Twenty-four hour-old...”

Line 42: consider replacing “activity” by “epidemic”

Response: changed as suggested, thanks!

Line 61: “as similar to”

Response: changed as suggested, thanks!

Line 91: “We evaluated the vector competence of *Cx. interfor* and *Cx. saltanensis* against SLEV from central Argentina compared to the natural vector, *Cx. p. quinquefasciatus*”. This sentence suggests that *Cx. interior* and *Cx. saltanensis* are “artificial” vectors. However, as stated in the introduction they can be naturally infected. Consider replacing “the natural vector” by “the primary urban vector”

Response: replace "the natural vector" with " the primary urban vector”

Line 82: I do not really understand what “horizontal transmission” refers to here (sexual transmission between mosquitoes?)

Response: “Horizontal transmission” is transference of arbovirus from infective mosquitoes to vertebrate hosts during blood feeding (Clements, 2012). In this case, “horizontal transmission” is transference of SLEV from infective *Cx. p. quinquefasciatus* to chicks during blood feeding [8, 12].

Clements AN. 2012. “The biology of mosquitoes. Vol. III: transmission of viruses and interactions with bacteria”. Cabi, Wallingford. United Kingdom. 563 p.

[8]Diaz LA, Flores FS, Beranek M, *et al.* Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Córdoba, Argentina. *Trans R Soc Trop Med Hyg* 2013; 107: 332–334.

[12]Mitchell CJ, Monath TP & Sabbatini MS. Transmission of St. Louis encephalitis virus from Argentina by mosquitoes of the *Culex pipiens* (Diptera: Culicidae) Complex. *J Med Entomol* 1980; 17: 282–285

Table 1: replace Specie by Species

Response: changed as suggested, thanks!

Line 109: replace “are” by “and” in: “...emergence, are adults provided”

Response: changed as suggested, thanks!

Line 115: delete “than”

Response: changed as suggested, thanks!

Line 119: “of a infected Swiss albino” replace “a” by “an”

Response: changed as suggested, thanks!

Line 200: SLEV is shown replace “is” by “are”

Response: changed as suggested, thanks!

Line 200 to 204. This is unclear. Does this mean that (i) the different chicks used to perform the oral mosquito infection carried about the same virus titers and (ii) that this possible source of variability (different infection doses) did not affect the infection, dissemination and transmission rate?

Response: This question was responds above

Line 205: significantly difference by “different”

Response: changed as suggested, thanks!

Line 208-209: Viral loads (range=1.3-5.3 log₁₀ PFU/ml) were evaluated in 89% (31/35) of the three species mosquitoes. How did it vary among the three mosquito species? Was it different between species?

Response: Number of samples which was quantified is not sufficient to indicate distributions, but that the trends were bimodal for *Cx. interfor* (2.4-5.3 log₁₀ PFU), *Cx. p. quinquefasciatus* "symmetric" (1.3-2.9 log₁₀ PFU) and asymmetric to the left in *Cx. saltanensis* (3.8-5.1 log₁₀ PFU). Figure below:

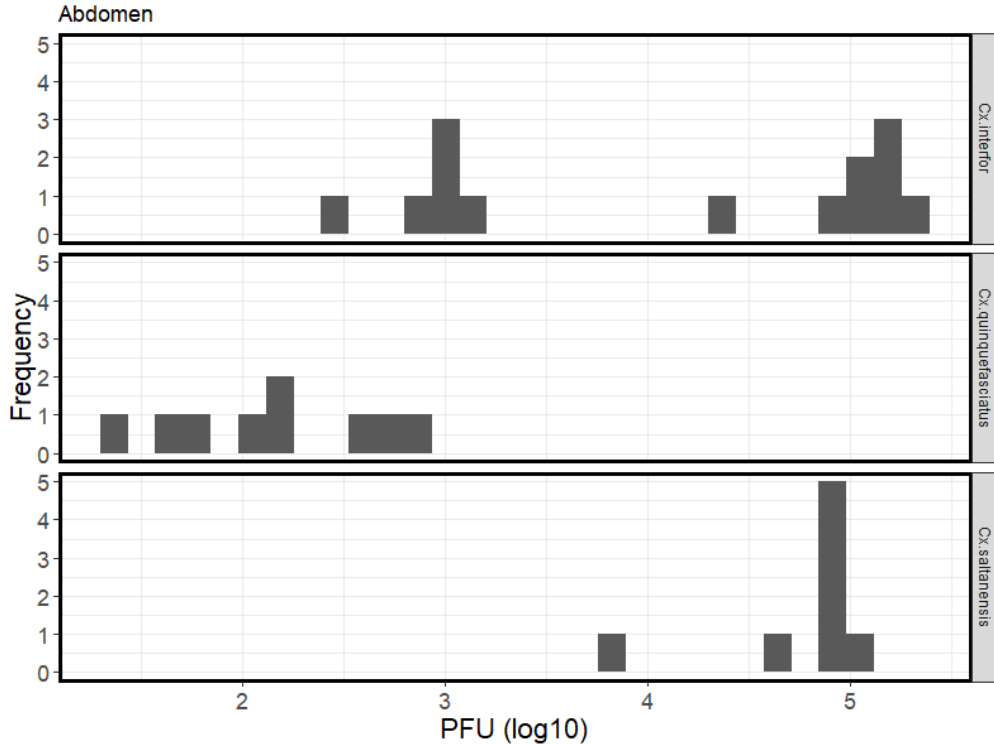


Figure: *Culex interfor*, *Cx. p. quinquefasciatus* and *Cx. saltanensis* abdomens positives frequency for SLEV with their PFU log₁₀.

Line 216: “These results represent a potential midgut barrier in *Cx. p. quinquefasciatus*” consider changing by “These results suggest the possible existence of a midgut barrier to SLEV in *Cx. p. quinquefasciatus*”

Response: changed as suggested, thanks!

Line 217-218: “Viral load ranged from 1.0-5.4 log₁₀ PFU/ml for 76% (19/25) of the three species mosquitoes with viral dissemination”. How did it vary among the three species? Was it different between species?

Response: Number of samples which was quantified is not sufficient to indicate distributions. Among infected mosquitoes, dissemination was achieved in 100% of those individuals tested (8/8) for *Cx. saltanensis*, while are 71% (10/14) of *Cx. interfor* and 54% (7/13) of *Cx. p. quinquefasciatus* demonstrated disseminated infections. The trends were for *Cx. interfor* (1-5.4 log₁₀ PFU), *Cx. p. quinquefasciatus* (2.9 log₁₀ PFU) and in *Cx. saltanensis* (1.6-5.1 log₁₀ PFU). Figure below:

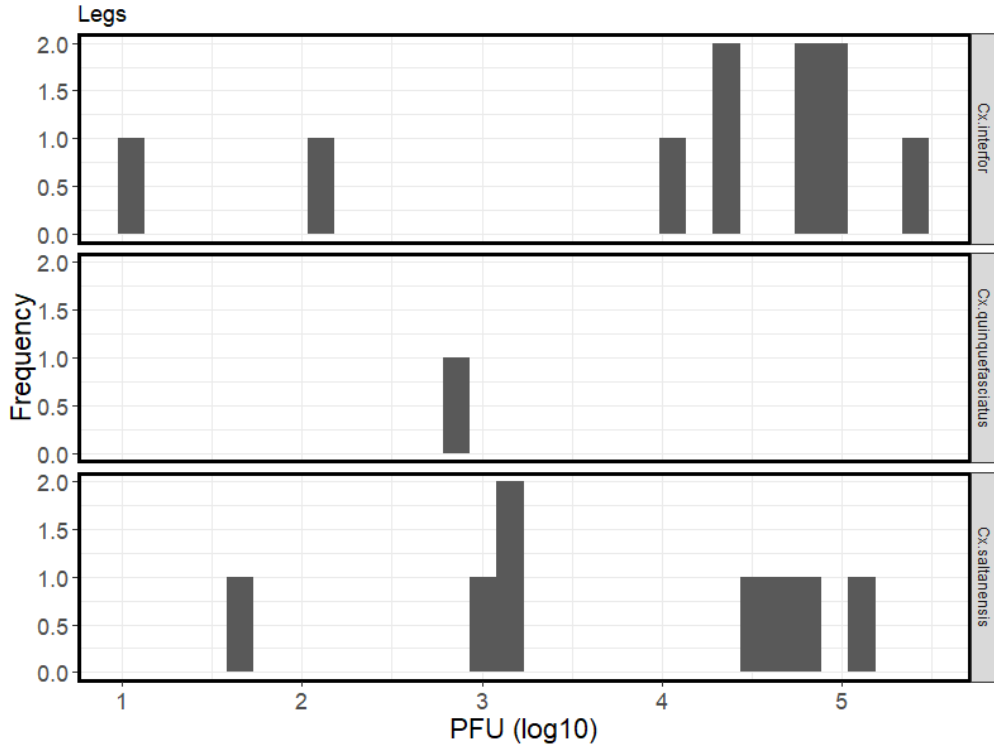


Figure: *Culex interfor*, *Cx. p. quinquefasciatus* and *Cx. saltanensis* legs positives frequency for SLEV with their PFU log₁₀.

Lines 224-225: The saliva viral load range was 1.1-2.3 log₁₀ PFU/ml in *Cx. saltanensis* and *Cx. interfor* (5/7, 71%) How did it vary among the three species? Was it different between species?

Response: Number of samples which was quantified is not sufficient to indicate distributions. Viral particles could only be quantified in saliva *Cx. interfor* (N = 5) and *Cx. saltanensis* (N = 2) but were not quantified in *Cx. p. quinquefasciatus* (N = 7). The trends were for *Cx. interfor* (1.1-2.2 log₁₀ PFU) and in *Cx. saltanensis* (1.8) log₁₀ PFU). Figure below:

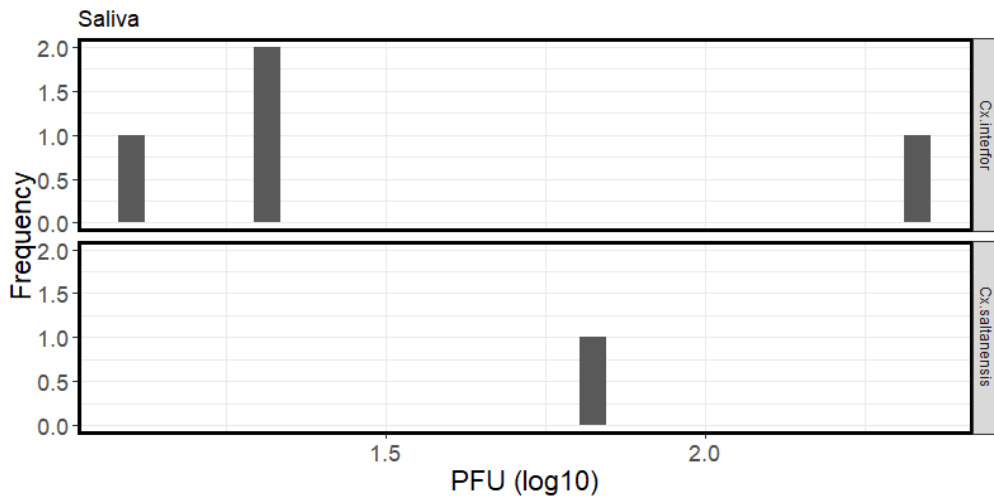


Figure: *Culex interfor*, *Cx. p. quinquefasciatus* and *Cx. saltanensis* saliva positives frequency for SLEV with their PFU log₁₀.

Line 235-236: "...they were not observed differences". Do you mean this was not statistically different? If yes please reword. In addition, increasing the sample size would perhaps make this stat different.

Response: changed as suggested. ..."They were not observed statistical differences, however, the number of tested mosquitoes was low, thus our results are not conclusive.

Line 246-247: ...of SLEV since (100%, 8/8) of the infected mosquitoes demonstrated disseminated virus, transmission, while only 25% (2/8) transmitted it". Consider deleting the word "transmission"

Response: changed as suggested, thanks!

Lines 274-276: "Furthermore, we were able to obtain an approximation of the MIT and EIP for *Cx. p. quinquefasciatus*, *Cx. interfor* and *Cx. saltanensis* of 2.9, 3.5 and 3.2 log₁₀ PFU/ml, respectively, because the infected mosquitoes transmitted SLEV 14 days after infection". MIT and EIP (even rough values) cannot be derived from this experiment. This would require (i) infecting the mosquitoes with a wide range of viremia (and look at the threshold above which mosquitoes become infected) and (ii) dissect mosquitoes at several time points. Perhaps the EIP of this viral strain is 2 days, we simply cannot tell until mosquitoes are dissected and checked for the presence of virus at this time point.

Response: changed as suggested. We deleted "Furthermore, we were able to obtain an approximation of the MIT and EIP for *Cx. p. quinquefasciatus*, *Cx. interfor* and *Cx. saltanensis* of 2.9, 3.5 and 3.2 log₁₀ PFU/ml, respectively, because the infected mosquitoes transmitted SLEV 14 days after infection".

Reviewed by anonymous reviewer, 2019-09-12

This study compares the efficacy of three different species of mosquitoes from the *Culex* genus as vectors of St Louis encephalitis virus (SLEV). *Culex saltanensis* and *Culex interfor* are thought to be new vectors, whereas *Culex pipiens quinquefasciatus* is considered the established and most common vector. Mosquitoes of each species were inoculated with the virus, and viral presence confirmed in the haemocoel, legs and salivary glands. Presence of the virus in the salivary glands was taken to mean that transmission could take place. They found that all 3 species became infected with SLEV, with no differences in levels detected in the haemocoel and salivary glands. Thus, they conclude that all species may be competent vectors.

Response: Virus was detected in abdomens (Infection rate), not in the hemocel and in expectorated saliva (transmission rate) and not in the salivary glands. Infection rates were defined as the numbers of positive mosquito abdomens of the total number of blood feed mosquitoes. Dissemination rates were calculated as the number of mosquitoes with positive legs out of the number of mosquitoes fed analyzed. Transmission rates were determined by the number of mosquitoes with positive saliva of the total number of mosquitoes that blood fed (Line 179-184). Mosquito abdomens, legs, and salivary secretions were assayed for infection by plaque assay on African Green monkey kidney (Vero) cells. Infection, dissemination, and transmission are defined as the presence of infectious SLEV in mosquito abdomens, legs, and salivary secretions, respectively. We conclude that the three species are competent laboratory vectors, because they acquired infections and transmit the SLEV in saliva.

I enjoyed reading the manuscript and think the subject is suitable for PCI Entomology. However, revision is required before it can be recommended. Please see below recommendations that I hope will improve the clarity of the manuscript.

Response: changed as suggested, thanks!

I think the Introduction needs more information describing the ecology of the virus, notably that it has many vertebrate hosts and *Culex* vectors. As presented, it seems that *Cx. saltanensis* and *Cx. interfor* are new potential vectors, but then later in the Discussion it is revealed that there are actually many known *Culex* vectors. Another concern is that the study doesn't measure actual transmission. This is fine, but please provide information about whether presence in the salivary glands means that transmission can occur. If viral particles from the salivary glands infect cell culture, does this mean that they can also be transmitted in to the blood. Describe somewhere about viral replication in the mosquito.

Response: changed as suggested. We added “SLEV is an endemic neurotropic flavivirus in temperate and subtropical areas of the New World that is maintained between multiple avian hosts and *Culex* vectors, although incidental infections are possible in humans and other mammals, which are typically dead-end hosts [4]”.

Culex interfor and *Cx. saltanensis* are new potential vectors of SLEV, but others *Culex* species or others genera mosquitoes can play in SLEV transmission. For example, *Mansonia titillans* (Beranek et al., 2018). We quantified viral particles of SLEV on the expectorated saliva the three *Culex* species studied as described by (Richards et al., 2012; Anderson et al., 2010; Ebel et al., 2005), but salivary glands were not dissected (line 145-147). Then, transmission rate was determined by the number of mosquitoes with positive saliva of the total number of mosquitoes that blood fed (Line 182-184). Mosquito salivary secretions were assayed for infection by plaque assay on African Green monkey kidney (Vero) cells. Viral replication in mosquito, were described in line 61-71 (This question was responds above).

Beranek MD, Gallardo R, Almirón WR *et al.* First detection of *Mansonia titillans* (Diptera: Culicidae) infected with St. Louis encephalitis virus (Flaviviridae: Flavivirus) and Bunyamwera serogroup (Peribunyaviridae: Orthobunyavirus) in Argentina. *J Vector Ecol* 2018. 43: 340-343

There are a number of grammatical mistakes throughout the ms that need to be corrected.

Response: changed as suggested, thanks!

Abstract

Line 32: Put the species name instead of ‘a recognized vector’.

Response: changed as suggested, thanks!

Line 34: Does the strain name need to be here? If so maybe add the importance of the strain, namely that it is the same strain detected in the US and Argentina, and responsible for human disease.

Response: changed as suggested. We added “...SLEV CbaAr-4005, it is the same strain detected in the US and Argentina, and responsible for human disease.”

Introduction

I would start the introduction with a more general paragraph setting the scene in the context of parasite-vector interactions being more or less specialist/generalist, and consequences for parasite transmission/epidemiology, before discussing the specific system.

Response: We modify the paragraph according to revisions “Infectious diseases caused by vector-borne pathogens constitute health and economic problems worldwide [1]. Mosquitoes are an important group of arthropod vectors. Due to the hematophagous habit of females, mosquito species are competent vectors of infectious agents, including viruses (arthropod-borne viruses; ‘arboviruses’) [2]. Arbovirus is maintained by biologic transmission among vectors and hosts. Sometimes this biological transmission is specific and includes one vector and host species such as Chikungunya (CHIKV), Dengue (DENV), urban Yellow Fever

(YFV) and Zika viruses (ZIKV). However, most of the arbovirus is generalist and they use many vectors and hosts species such as St. Louis encephalitis virus (SLEV) and West Nile virus (WNV) [3]. The emergence and reemergence of diseases caused by arbovirus are a global phenomenon, in particular, those caused by CHIKV, DENV, WNV and ZIKV [1].”

[1]Gould E, Pettersson J, Higgse S *et al.* Emerging arboviruses: Why today? *One Health* 2017. 4: 1-13.

[2]Agarwal A, Parida M, Dash PK. Impact of transmission cycles and vector competence on global expansion and emergence of arboviruses. *Rev Med Virol* 2017: e1941.

[3]Diaz LA, Flores FS, Quaglia A *et al.* Intertwined arbovirus transmission activity: Reassessing the transmission cycle paradigm. *Front Physiol* 2013: 3: 493.

Lines 49–60: Add in this paragraph whether detection in humans is recent. Is transmission possible from humans, or are they just a spillover host? The second half of the paragraph could be more concise, stating that re-emergences have occurred in the US (add the year) and Argentina (2002), with a particularly large outbreak in Cordoba City, and that the same strain is probably responsible.

Response: We modify the paragraph according to revisions “SLEV is an endemic neurotropic flavivirus in temperate and subtropical areas of the New World that is maintained between multiple avian hosts and *Culex* vectors, although incidental infections are possible in humans and other mammals, which are typically dead-end hosts [4]. In 2002, SLEV reemerged in the central area of Argentina and southern Brazil causing neurological diseases in humans. In 2005, the first outbreak occurred in Córdoba City with 47 confirmed cases and nine fatalities. After the 2005 outbreak, additional SLEV outbreaks in Argentina occurred in Parana (2006), Buenos Aires (2010), and San Juan (2011) [5]. Factors that promoted this emergence in Argentina include the introduction of a more virulent SLEV strain into a highly susceptible avian hosts community along with possible land use changes (urbanization, agriculture) [5]. Phylogenetic analyses indicate that the emerging SLEV in US (2015) is related to the epidemic strains isolated during a human encephalitis outbreak in Córdoba, Argentina, in 2005 [5].

Lines 61–65: These sentences should be referenced.

Response: We added “[6]”

[6] Franz AWE, Kantor AM, Passarelli AL *et al.* Tissue barriers to arbovirus infection in mosquitoes. *Viruses* 2015: 7: 3741–3767.

Lines 68–71: This repeats the previous 3 sentences. Just state these terms whilst describing these stages the first time round (lines 65–68).

Response: changed as suggested. We added “The virus-vector interaction is essential for its effective transmission and is influenced by both viral and vector components [6]. A prerequisite for the systemic and persistent infection of the vector is the pathogen being able to overcome all tissue barriers [6, 7]. There are barriers in mosquitoes that affect the likelihood for viral transmission after ingesting an infectious bloodmeal [7]. Viruses enter the midgut epithelial cells (midgut infection barrier-MIB), replicate, exit the cells (midgut escape barrier-MEB), and travel through the hemolymph-filled hemocoel to the salivary glands (salivary gland infection barrier-SGIB), where they again replicate and reside followed by the subsequent transmission through the saliva (salivary gland escape barrier-SGEB).

Line 74: Do you mean the avian host here?

Response: changed as suggested. We added “...avian host availability and avian host immunity [7]”.

Line 72: These measures are proportions, not rates. Please change throughout the manuscript.

Response: changed as suggested, thanks!

Lines 79–81: Were these SLEV infected mosquitoes detected during this period?

Response: We added “*Culex p. quinquefasciatus* have been detected during periods without reports of clinical disease symptoms in Santa Fe province between 1978-1983 [10] and Córdoba between 2001-2004 [11]”.

Lines 81–84: This sentence is unclear. Population abundance of what? Horizontal transmission between avian or mosquito hosts?

Response: Refers to population abundance female *Cx. p. quinquefasciatus* in Córdoba City [9] and horizontal transmission (in lab) is transference of SLEV from infected adult female of *Cx. p. quinquefasciatus* to chicks during blood feeding [8, 12].

[8] Diaz LA, Flores FS, Beranek M *et al.* Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Córdoba, Argentina. *Trans R Soc Trop Med Hyg* 2013; 107: 332–334.

[9] Diaz LA, Ré V, Almirón W *et al.* Genotype III Saint Louis encephalitis virus outbreak, Argentina, 2005. *Emerg Infect Dis* 2006; 128: 1752–1754.

[12] Mitchell CJ, Monath TP & Sabattini MS. Transmission of St. Louis encephalitis virus from Argentina by mosquitoes of the *Culex pipiens* (Diptera: Culicidae) Complex. *J Med Entomol* 1980; 17: 282–285.

Materials and Methods

Line 101: Did you track how many adult females came from each raft?

Response: We did not track adult females from each raft.

Line 115: This sentence should only describe the viral strain used. It is confusing to talk about adult female mosquitoes here.

Response: changed as suggested. We deleted “...a strain isolated from *Cx. p. quinquefasciatus* mosquitoes during the 2005 human encephalitis outbreak in Córdoba City”.

Line 122: Shouldn't the description of viral titration be a new paragraph. Isn't this how you quantified virus in the mosquitoes?

Response: changed as suggested. Mosquito abdomens, legs, and salivary secretions were assayed for infection by plaque assay on African Green monkey kidney (Vero) cells.

Line 133: How many chicks?

Response: We did not use 20 infected chicks; only used 3 infected chicks, one for each *Culex* species studied. “There was a narrow range of viremia during blood feeding (*Cx. p. quinquefasciatus*=2.9 log₁₀ PFU/ml, *Cx. interfor*=3.5 log₁₀ PFU/ml and *Cx. saltanensis*=3.2 log₁₀ PFU/ml). We added in line 133 “Twenty-four hour-old...”

Line 152: What were the cellular and viral controls?

Response: Virus and cell controls were included in duplicate in viral plaque assay in VERO cell monolayer.

Lines 181–182: State why dissemination rates are important. Does this give a measure of within-vector replication?

Response: In our study dissemination rate was important to determine if the virus escaped from the midgut of infected mosquitoes. Before transmission virus in saliva, must first spread by body (in this case we use the legs of the SLEV positive individuals to determine if the virus had left the midgut). Franz et al., (2015) “Following dissemination from the midgut, it has been hypothesized that an arbovirus requires (1) a means to amplify after its escape from midgut epithelial cells, so that it can infect the salivary glands efficiently, and (2) a vehicle to ensure dissemination from the midgut to the salivary glands. Following escape from the midgut, arboviruses typically disseminate to secondary tissues such as fat body, hemocytes, and nerve tissue. Once the salivary glands of the mosquito become infected, virus is transmitted along with the saliva”.

Franz AWE, Kantor AM, Passarelli AL *et al.* Tissue barriers to arbovirus infection in mosquitoes. *Viruses* 2015; 7: 3741–3767.

Lines 182–184: If viral particles are found in the salivary glands, are you sure that the virus can be transmitted? In some species of plant virus, transmission to a non-competent arthropod vector can result in viral particles being found throughout their body, but no transmission to a new host.

Response: We quantified viral particles of SLEV on the expectorated saliva the three *Culex* species studied as described by (Richards et al., 2012; Anderson et al., 2010; Ebel et al., 2005), but salivary glands were not dissected (line 145-147). Then, transmission rate was determined by the number of mosquitoes with positive saliva of the total number of mosquitoes that blood fed (Line 182-184). Mosquito salivary secretions were assayed for infection by plaque assay on African Green monkey kidney (Vero) cells. Viral replication in mosquito, were described in line 61-71 (This question was responds above).

Lines 186–187: This sentence is not clear.

Response: changed as suggested. We rewrite sentence “*Culex p. quinquefasciatus* was a reference species as the assayed population was susceptible to SLEV [8]”.

[8] Diaz LA, Flores FS, Beranek M, *et al.* Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Córdoba, Argentina. *Trans R Soc Trop Med Hyg* 2013; 107: 332–334

Line 188: If there were multiple feeding trials this should be included in the statistical model as a random factor. This will account for variance in viremia levels between feeding trials. Why not do a separate model looking to see how viremia levels change in the haemocoel, legs and salivary glands changes for the different species? Also, if you can track which mosquito was fed on which chick, chick should also be included in the statistical models as a random factor.

Response: We did not use 20 infected chicks; only used 3 infected chicks, one for each *Culex* species studied. “There was a narrow range of viremia during blood feeding (*Cx. p. quinquefasciatus*=2.9 log₁₀ PFU/ml, *Cx. interfor*=3.5 log₁₀ PFU/ml and *Cx. saltanensis*=3.2 log₁₀ PFU/ml”). Differences in viremia levels (range: 2.4-3.4 log₁₀ PFU/ml) between feeding trials could be a source of variation confounding the outcomes analyzed, therefore viremia level was added as a co-variable (line 187-190).

Results

Lines 201-202: Include a measure of variance when you present means such as confidence intervals or standard errors. Please correct this throughout the manuscript.

Response: changed as suggested, thanks!

Line 208: Add that this refers to Figure 1. This needs to be corrected elsewhere in the manuscript.

Response: changed as suggested, thanks!

Lines 208–209, lines 216–218 and lines 224–225: Why were viral loads only measured for a subset of infected mosquitoes? Also, just stating that they were measured tells us nothing. It would be interesting to do a statistical model to see how the viral loads differ between the different species.

Response: In some positives sample infected mosquitoes, lysis plaque was not confluent and not possible to measure. Number of samples which was quantified was not sufficient to indicate stat distributions (This question was responds above).

Line 210: Please show the results for the main effect of species in the Dissemination model, not just the pairwise comparisons.

Response: Odds Ratio is a way to represent the size of effect (ratio between proportions).

Lines 210–226: It would also be more informative to measure dissemination and transmission as a proportion of those that became infected, not the total number fed on infected chicks.

Response: We use mosquitoes fed and not infected to calculate the rates of dissemination and transmission, because we are concerned about the ecological meaning of the susceptibility to transmission for a given mosquito species than virogenesis features they provide more real ecological information with respect to what happens with natural populations.

“Moreover, we presented in Results section, dissemination and transmission rates as a proportion of mosquitoes infected and not of the number of mosquitoes fed analyzed (Lines 213-215 and 221-223)“.

Line 216: Why does this suggest a midgut barrier for *Cx. p. quinquefasciatus* and not *Cx. saltanensis*? Please elaborate, but this information should be in the Discussion not the Results.

Response: This result derived of our study and it could the response in the difference found between the species of mosquitoes infected with SLEV. Among infected mosquitoes, dissemination was achieved in 100% of those individuals tested (8/8) for *Cx. saltanensis*, while are 71% (10/14) of *Cx. interfor* and 54% (7/13) of *Cx. p. quinquefasciatus* demonstrated disseminated infections. This is a measure of the potential for midgut barriers in *Cx. p. quinquefasciatus*. See Figure 1

Line 223–224: This information about a potential salivary gland barrier should be in the Discussion.

Response: This result derived of our study and it could the response in the difference found between the species of mosquitoes infected with SLEV. All *Cx. p. quinquefasciatus* females with disseminated infections demonstrated SLEV in their saliva (7/7); this rate was only 50% (5/10) in *Cx. interfor* and 25% (2/8) in *Cx. saltanensis*, indicating a potential salivary gland barrier in both *Cx. interfor* and *Cx. saltanensis*. This is a measure of the potential salivary gland barrier in *Cx. saltanensis* and *Cx. interfor*. See Figure 1

Discussion

Lines 233–236: This sentence doesn't make sense.

Response: The sentence attempted to claim “would have a greater chance...” regarding that the observed rates of infection and dissemination followed a decreasing order between *Cx. saltanensis* (67%) and *Cx. interfor* (infection = 50%, dissemination = 40%) and ending in *Cx. p. quinquefasciatus* (infection = 33%, dissemination = 18%).

Lines 240–242: It would be interesting to elaborate here and discuss in more detail the midgut and salivary gland barriers, and how they can prevent the transmission of other parasites by mosquitoes.

Response: changed as suggested, thanks!

Line 247 and line 252: It is misleading to say that the virus was transmitted. This study only measures the potential for transmission by showing that the virus could migrate to the salivary glands in the vector.

Response: We quantified viral particles of SLEV on the expectorated saliva the three *Culex* species studied as described by (Richards et al., 2012; Anderson et al., 2010; Ebel et al., 2005), but salivary glands were not dissected (line 145-147). Then, transmission rate was determined by the number of mosquitoes with positive saliva of the total number of mosquitoes that blood fed (Line 182-184). Mosquito salivary secretions were assayed for infection by plaque assay on African Green monkey kidney (Vero) cells. (This question was responds above).

Lines 247–250: Did this study actually measure transmission, or just the presence of the virus in the salivary glands?

Response: Turell et al (2000) determined a low transmission rate even after intrathoracic inoculation, indicating the presence of a salivary gland barrier in these mosquito species. Section Materials and methods, “some mosquitoes were inoculated intrathoracically with virus to determine transmission rates for individuals with a disseminated viral infection. To determine if the mosquitoes could transmit virus by bite, mosquitoes were allowed to feed on susceptible hamsters either individually or in small groups of two to five mosquitoes. Because VEE virus infection is consistently fatal to hamsters, death of these animals was used to indicate virus transmission. Transmission was verified by isolating virus from brain tissue”.

Turell MJ, Jones JW, Sardelis MR *et al.* Vector competence of Peruvian mosquitoes (Diptera: Culicidae) for epizootic and enzootic strains of Venezuelan equine encephalomyelitis virus. *J Med Entomol* 2000; 37: 835–839.

Lines 259–264: Why do your results corroborate the results of Diaz et al when you find much lower infection rate.

Response: Diaz et al (2013) demonstrated that *Cx. quinquefasciatus* mosquitoes from Córdoba City fed on viremic chicks became infected and able to transmit SLEV strains under laboratory conditions. This study did not determine vector competence, but in our study vector competence assays completed for same population of *Cx. p. quinquefasciatus* from Córdoba City. This difference could be related to the lower viremia level that the mosquitoes were exposed to in our assay (2.9 log₁₀ PFU/ml vs 5.2 log₁₀ PFU/ml).

Diaz LA, Flores FS, Beranek M *et al.* Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Córdoba, Argentina. *Trans R Soc Trop Med Hyg* 2013; 107: 332–334.

Lines 266–267: How are your measures of dissemination and transmission different, and which is better?

Response: Comparing our data *Cx. p. quinquefasciatus* with those obtained by Mitchell et al (1980). These authors calculated the rates of dissemination and transmission using SLEV infected mosquitoes and not fed mosquitoes. Our results were not that different than those of Mitchell et al. He found 19/21 INFECTED mosquitoes transmitted virus and we found 7/13, and these rates are barely significantly different, Fisher’s Exact Test, p=0.03, not all that meaningful. Also, not only are infection rates affected by virus dose, but so are dissemination rates in infected mosquitoes, i.e., a higher percentage of the infected mosquitoes will have a disseminated infection if the mosquitoes ingested a higher dose of virus. Mitchell et al. used a higher infectious dose (4.1-4.8 log₁₀ PFU/ml vs our study, 2.9 log₁₀ PFU/ml), so their dissemination rate would have been higher, and we found 100% of disseminated mosquitoes (*Cx. p. quinquefasciatus*) transmitted virus. Thus, we consider we results and Mitchells to be very similar.

Mitchell CJ, Monath TP & Sabattini MS. Transmission of St. Louis encephalitis virus from Argentina by mosquitoes of the *Culex pipiens* (Diptera: Culicidae) Complex. *J Med Entomol* 1980; 17: 282–285.

Lines 272–276: What are the viremia levels presented for the MIT here? Levels measured on day 14? All you can really say about EIP is that it is less than 14 days.

Response: changed as suggested. We deleted “Furthermore, we were able to obtain an approximation of the MIT and EIP for *Cx. p. quinquefasciatus*, *Cx. interfor* and *Cx. saltanensis* of 2.9, 3.5 and 3.2 log₁₀ PFU/ml, respectively, because the infected mosquitoes transmitted SLEV 14 days after infection”.

Line 282: This information about mortality and low feeding success should be described in more detail in the methods.

Response: changed as suggested, thanks!

Lines 288–291: As far as I can see there is no evidence showing that *Cx. interfor* and *Cx. saltanensis* were not previously vectors? Or have they only recently been identified as being infected? Did anyone look before?

Response: *Culex saltanensis* and *Cx. interfor* participate in the maintenance of SLEV and could assist in the spillover of SLEV to humans. In 2004, prior to the outbreak of encephalitis in Córdoba City, SLEV infected *Cx. interfor* were detected [11]. In 2010, there were small outbreaks of SLEV in provinces, e.g., Buenos Aires, Córdoba and San Juan [37]. Not long thereafter, SLEV infected *Cx. saltanensis* were detected for first time in Córdoba City [14]. The presence of naturally SLEV-infected *Culex interfor* and *Culex saltanensis*, dominant species in urban-vegetated sub-assemblage that frequently feed on competent hosts, suggest they could participate as vectors in the transmission network of SLEV [3, 8, 9, 14].

[3]Diaz LA, Flores FS, Quaglia A *et al.* Intertwined arbovirus transmission activity: Reassessing the transmission cycle paradigm. *Front Physiol* 2013; 3: 493.

[8]Diaz LA, Flores FS, Beranek M, *et al.* Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Córdoba, Argentina. *Trans R Soc Trop Med Hyg* 2013; 107: 332–334.

[9]Diaz LA, Ré V, Almirón W *et al.* Genotype III Saint Louis encephalitis virus outbreak, Argentina, 2005. *Emerg Infect Dis* 2006; 128: 1752–1754.

[11]Diaz LA, Albrieu Llinás G, Vázquez A *et al.* Silent circulation of St Louis encephalitis virus prior to an encephalitis outbreak in Córdoba, Argentina (2005). *PLoS Negl Trop Dis* 2012; 6: 1489.

[14]Batallán GP. Binomía de *Culex interfor* Dyar en Córdoba, Argentina. [theses]. Córdoba: Universidad Nacional de Córdoba; 2013. 197 p.

[37]Vergara Cid C, Spinsanti L, Rivarola M *et al.* Detección de infecciones humanas por *Flavivirus* en la ciudad de Córdoba durante el año 2010. *Rev Argent Microbiol* 2011; 43: 37.

Lines 297–308: This information should be presented in the Introduction.

Response: This information was presented in Introduction in lines 84-87. "The presence of naturally SLEV-infected *Culex interfor* and *Culex saltanensis*, dominant species in urban-vegetated sub-assemblage that frequently feed on competent hosts, suggest they could participate as vectors in the transmission network of SLEV [3, 8, 9, 14].

Lines 297–304, we intend to relate our results together with ecological importance of *Cx. interfor* and *Cx. saltanensis* in the maintenance and transmission of SLEV in Córdoba City.

Lines 304-308, we compare our results "is transmitted by multiple sympatric *Culex* spp., and that both *Cx.*

saltanensis and *Cx. interfor* can be considered potential vectors of SLEV" with what happens in the US, where there are different mosquito species as primary vectors transmitting SLEV in different geographical areas [4].

[3]Diaz LA, Flores FS, Quaglia A *et al.* Intertwined arbovirus transmission activity: Reassessing the transmission cycle paradigm. *Front Physiol* 2013. 3: 493.

[8]Diaz LA, Flores FS, Beranek M *et al.* Transmission of endemic St Louis encephalitis virus strains by local *Culex quinquefasciatus* populations in Córdoba, Argentina. *Trans R Soc Trop Med Hyg* 2013; 107: 332-334.

[9]Diaz LA, Ré V, Almirón W *et al.* Genotype III Saint Louis encephalitis virus outbreak, Argentina, 2005. *Emerg Infect Dis* 2006; 128: 1752-1754.

[14]Batallán GP. Binomía de *Culex interfor* Dyar en Córdoba, Argentina. [theses]. Córdoba: Universidad Nacional de Córdoba; 2013. 197 p.

[4]Reisen WK. Epidemiology of St. Louis encephalitis virus. *Adv Virus Res* 2003; 61: 139–184.

Lines 309–310: This information should also be presented in the Introduction.

Response: This information was presented in the Introduction and modified suggested recommenders. "SLEV is an endemic neurotropic flavivirus in temperate and subtropical areas of the New World that is maintained between multiple avian hosts and *Culex* vectors, although incidental infections are possible in humans and other mammals, which are typically dead-end hosts [4] (Lines 49-51).

Table 1: You can put most of the information about the different columns, except about the egg rafts, as the column headings. For example, 'no. of egg rafts per species', 'total no. of females fed on chicks', 'no. of engorged females', and 'no. of SLEV positive females'. Also, why were so few, and different numbers of females for each species retained for infection?

Response: changed as suggested. Different numbers of females, because at the time vector competence experiment were conducted there were not breeding protocols in lab and it was very difficult to keep them alive until 14 days. In this moment, we are improving the breeding techniques and the survival of adult females of *Cx. interfor* and *Cx. saltanensis* for future experiment.

Table 2 is difficult to read. Change the stating exactly what is shown. I think this would be something like 'Vector competence of Cp, Ci and Cs for SLEV measured as infection, dissemination and transmission. Table shows the proportion of mosquitoes positive for the virus and viremia levels in the haemocoel, legs and salivary glands of each species. The table would be much simpler, and it would be easier to compare among species, if the species were the columns and infection, transmission and dissemination the rows. Under each species you could have a sub-column for N, Rate (Proportion) and Viral load.

Response: changed as suggested, thanks!

Figure 1 is also unclear. It would be simpler if you had 3 panels, 1 for each species. Or if you want to have only 1 panel, change it so that each of the colored bars is a different species and infection, dissemination and transmission are on the x-axis. It is also not clear which pairwise comparison the dashed line showing the odds ratio refers to.

Response: Figure was 1 show species mosquito in x-axis and different colors for infection, dissemination and transmission rates for SLEV because it allows us to understand best the possible potential midgut barrier in *Cx. p. quinquefasciatus* and a potential salivary gland barrier in *Cx. interfor* and *Cx. saltanensis*. Dashed line

indicates statistical difference on dissemination rate for *Cx. saltanensis* in relation to *Cx. p. quinquefasciatus* (OR=8.9; p=0.01).