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Detección de arbovirus en mosquitos *Aedes aegypti* en dos comunidades de Manabí, Ecuador

Disertación previa a la obtención del título de Microbiólogo

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CERTIFICACIÓN

Yo Anita Gabriela Villacís Salazar PhD, certifico que la disertación de Microbiología del estudiante Alvaro Steeven Wilca Cepeda ha sido concluida de conformidad con las normas establecidas; por lo tanto, puede ser presentada para la calificación.

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Arbovirus detection in *Aedes aegypti* mosquitoes within two communities in Manabí, Ecuador employing three molecular techniques.

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Arbovirus detection in *Aedes aegypti* mosquitoes within two communities in Manabí, Ecuador employing three molecular techniques.

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Abstract

The arboviruses transmitted by *Aedes aegypti* cause a substantial problem in Ecuador, since they can be persistent, emerging, and re-emerging. During the SARS COV-19 pandemic, these diseases took a backseat. As the arboviruses are transmitted by *Aedes argypti*, the present study sought the molecular characterization of the different species of arboviruses within this species of mosquito collected in two communities, Caja Fuego and San Gregorio, in the province of Manabí. For this, i) a total of 468 mosquitoes were collected, 385 mosquitoes in the rural community of Caja Fuego and 83 in the marginal urban area of San Gregorio. ii) The samples were transported with all permits and biosafety regulations to CISeAL. iii) The samples were homogenized following the TRI Reagent® protocol. iv) Three methodologies were applied (end-point PCR, RT-PCR and Sanger sequencing), and vi) different arboviruses were identified. Regarding the results, flavivirus and alphavirus were identified. For flavivirus, of a total of 22 samples, 18 were positive, and of these, the final point PCR analysis was performed for Dengue and Zika. Nevertheless, no identification of arbovirus was obtained with RT-PCR, but 2 Zika positives were detected with Sanger sequencing. In contrast, there were no officially reported cases of Zika in 2023. This demonstrates a possible risk of infection in the human populations who live in the Caja Fuego and San Gregorio communities. It is recommended that a vigilance campaign be implemented in this zone, in collaboration with the Ecuadorian Ministry of Public Health.

Keywords: *Aedes aegypti,* Dengue, Zika, communities, Manabí, Ecuador.

Introduction

Arboviruses are a virus group belonging to the *Togaviridae* family, which are characterized by their spherical form and genomic RNA. They have the capacity to be transmitted by vectors such as arthropods, mainly by the genus *Aedes* mosquitoes. Viruses such as Zika (ZIKV), Dengue (DENV) and Chikungunya (CHIKV), cause high rates of morbidity and mortality worldwide. *Aedes aegypti*'s life cycle is developed in four phases: egg, larvae, pupa, and adult, which is why it is considered a holometabolous species. However, the epidemiology of the arboviruses is complex due to diverse factors such as: i) adult *A. aegypti* females being exclusively responsible for the transmission of the arboviruses through human blood consumption (Velandia-Romero et al., 2017), ii) socioeconomic circumstances (Alvarado-Prado & Nieto, 2019), iii) climate change (Alvarado-Prado & Nieto, 2019), iv) natural disasters (Alvarado-Prado & Nieto, 2019) and v) human mobilizations, as these lead to an increase in the mosquito population (Alvarado-Prado & Nieto, 2019). For this reason, *A. aegypti* has been able to expand to new geographical locations. These variations have resulted in the ability of this vector to colonize new habitats in which it had not been previously present (Alvarado-Prado & Nieto, 2019).

The epidemiological importance of *A. aegypti* resides not only in its capacity to host the different types of Dengue, Zika and Chikungunya viruses, but in the fact that they are most common in tropical and subtropical zones within America, Africa, Asia, and Oceania. Transmission is also significant in areas where these mosquitoes are not commonly found, but still inhabit (Betancourt-Cravioto & Falcón-Lezama, 2020).

This may pose a challenge for the healthcare systems and could require control measures and effective preventative strategies. It is also necessary to consider that an epidemic may have a high impact in a country's economy, decreasing its labor

productivity, tourism, and commerce in rural and urban areas, while increasing medical care costs (Castañeda- Porras & Zuleta-Dueñas, 2017).

According to Pan American Health Organization (PAHO), the transmission of diseases generated by Dengue, Zika and Chikungunya has increased in the Americas between 2013 and 2017. Additionally, there has been no significant control strategy implemented for the reduction of diseases such as hemorrhagic fever, dengue fevers, among others (WHO, 2023). Globally, the reported development of diseases linked to these arboviruses is 17% when compared to other diseases, and left untreated, represent 1 million deaths per year (Uribe-Álvarez & Félix, 2017).

Dengue is the arbovirus within Vector-Borne Diseases (VBD) that causes the majority of deaths in the Americas, with an outbreak frequency of 3 to 5 years (MSP, 2020b). There was an increase in the recorded cases of Dengue virus in the Americas in 2018. In 2019, Ecuador reported 8,419 cases of Dengue, of which 1,718 cases (20.47%) had warning signs. The lethality rate was 0.044% (MSP, 2015). In 2022, there were 16,017 notified, confirmed cases of Dengue. Of these, 14,133 (88.23%) were Dengue with no warning signs, 1,775 cases (11.08%) were Dengue with warning signs and 109 cases (0.68%) were severe Dengue, with circulating serotypes DENV-1 and DENV-2 (MSP, 2023b). Additionally, during 2014-2020 in Manabí, the rate of Dengue per 100,000 inhabitants was 84.61% (MSP, 2020a).

Similarly, since Zika's arrival to America in 2014, there has been an unusual increase in microcephaly. By 2016, there were 4,180 cases reported in Ecuador due to Zika-associated infections. The presence of Zika increased exponentially by 85% after the 2016 earthquake (Valero-Cedeño et al., 2020).

Finally, Chikungunya cases have been registered since 2013, giving origin to over 1.3 million infections in 43 countries within the American continent. In May 2015, in

Ecuador, Chikungunya registered a 25% increase of infections. There were 33,619 recorded cases in 2015; 1,860 cases in 2016; 196 in 2017; 8 in 2018; 2 in 2019 and 1 in 2020 (Valero-Cedeño et al., 2020). However, in 2022 viruses such as Chikungunya and Zika, which are transmitted by the same vector (*Aedes aegypti),* have not been notified (MSP, 2023b).

Currently, diverse endemic diseases have been left aside due to the pandemic generated by SARS COV-19. This has resulted in a lack of control and follow-up in other diseases, with the consequence of an increase in Dengue, Zika and Chikungunya cases. Additionally, climatic changes have favored the increase of the populations of the arbovirus vector (*Aedes aegypti*), which could imply an extensive spread of these viral diseases (Segarra & Rubio, 2017).

Previous studies based in Ecuador do not prioritize the analysis of mosquitoes in the area occupied by the diseases caused by arbovirus. Instead, they are more focused on the mosquitoes' geographical adaptability and their resistance to specialized chemicals utilized for their control. Some of these studies are: i) Seasonal and geographic variation in insecticide resistance in *Aedes aegypti* in southern Ecuador (Ryan et al., 2019) and ii) Resistance status of *Aedes aegypti* to deltamethrin, malathion, and temephos in Ecuador (Morales et al., n.d.).

Therefore, the aim of this study was the molecular characterization of the different species of arboviruses in *Aedes aegypti* collected in two communities, Caja Fuego and San Gregorio, in the Manabí province, using multiplex real-time RT-PCR. With these results the goal is to reduce the risk of infection in the Manabí province, and we expect that the gathered information raises awareness among the population of these endemic areas, regardless of their social group, which is directly involved in the arboviral

diseases (Santiago et al., 2018). This will allow the implementation of a surveillance system in the future, not just by the authorities, but also by society in general.

Material and Methods

Study area

The samples of this study were collected in the Manabí province, in two communities, (Caja Fuego - rural community) and Andrés Vera (San Gregorio - marginal urban) ranging from 65 to 400 meters above sea level (masl), located in a single county, Portoviejo (Fig. 1).

Manabí province has a tropical and subtropical climate, this province presents six vegetation zones: deciduous forest, semi-deciduous forest, low mountain green forest, cloud forest, dry-montane shrub forest and tropical savannah (Grijalva et al., 2017). In the rural communities, agriculture is the main economic activity with a predominance of sugar cane, oranges, bananas, yucca, corn and rice. In addition, some palms such as cade or tagua (*Phytelephas aequatorialis*) and coconuts are cultivated. Most of the walls and floors of the houses are constructed with bamboo cane “caña guadúa” (*Guadua angustifolia*) or wood, and the roofs are commonly made of cade palm fronds, with a few made of zinc (Black et al., 2007; Villacís et al., 2017). The houses within the marginal-urban communities usually have brick walls, cement floors and zinc roofs (USGP et al., 2021).

Mosquito collection

The mosquito, *Aedes aegypti,* collection was in May of 2023 during the rainy season. This was carried out with a collection permit (MAAE-DBI-CM-2021-0185) and mobilization permit (MAAE-CMARG-2020-0178). The selected houses were

georeferenced using a global positioning system, (a single GPS point), thus obtaining a unique identification code for each collection site. The mosquitoes were collected using i) electric aspirators, Prokopack, due to its suction power, lightweight (0.5-kg) low rate of power consumption (4.3 amps), and water resistance. A 4:3-inch flexible rubber coupling (PlumbQuick model P1056-43, Fernco Inc., Davison, MI) (Vazquez-Prokopec et al., 2009), and ii) manual aspirators through a filter-equipped mouthpiece, ensuring the mosquitoes remained in good condition. In addition, at the field, aided by a stereoscope, *A. aegypti* was identified, and the sex was determined. This process was carried out with the personnel of the Centro de Investigación para la Salud en América Latina (CISeAL) and Ecuadorian Ministry of Public Health (MSP).

Subsequently, the mosquitoes were grouped in sets of 10 and placed in tubes containing 1000µl of TRI Reagent® solution. It is worth noting that the tubes with mosquitoes were kept at optimal temperatures for further processing in the laboratories of the CISeAL.

RNA extraction in pool

For the homogenization of the mosquitoes, plastic pestles were used and care was taken to avoid leaving any residue or large pieces, following the recommendations of TRI Reagent® to prioritize obtaining a good amount of RNA. Three washes of the RNA pellets were performed. First, 200µl of chloroform (CHCL2) was added per 1000µl of Trizol, vigorously agitated, and incubated for 15 minutes at room temperature; afterwards, it was centrifuged for 15 minutes at 12,000 RPM, 4°C, and the supernatant was transferred to a new tube. Second, 800µl of isopropanol (C3H8O) was added per 100µl of the new supernatant, vortexed for a few minutes, and incubated for 30 minutes at -80°C; then, it was centrifuged for 30 minutes at 12,000 RPM, 4°C, resulting in a new

pellet. Third, the pellet was washed with 1000µl of 75% molecular ethanol (EtOH), passed through a vortex, and underwent a final centrifugation for 5 minutes at 7,500 RPM, 4°C. Finally, the pellet was re-suspended in 50µl of nuclease-free water. However, two aliquots were taken from the 50µl, one of 35µl, which was stored at -80°C, and another of 15µl at -20°C, for subsequent analysis (López-Rosero et al., in prep).

cDNA Synthesis

The extracted RNA samples were converted to cDNA by following the Promega-GoScript Reverse Transcription Kit protocol. This process was divided into two stages: First, a mixture of 2 µl of nuclease-free water, 1 µl of random primers, and 2 µl of RNA sample was created. This first mixture was heated for 5 mins at 70°C to linearize the RNA, then it was frozen for 5 mins at 4°C, and finally, the mixture was exposed to centrifugal force for 10 seconds at 7.500 RPM. Second, the retrotranscription mix was prepared. 6.5 µl of nuclease-free water were mixed with 4 µl of GoScript 5X reaction buffer, 2 µl of MgCl, mM, 1 µl of PCR nucleotide mix, 0.5 µl of RNAase, and 1 µl of GoScript reverse transcriptase.

Once the creation of the mixture was finalized, the retrotranscription mix was combined with the linearized RNA (final volume 20 µl). Afterwards, it was placed into the thermo-cycler with the following settings: 25°C for 5 mins, 42°C for 1 hour, and 70°C for 15 mins. After the cDNA was obtained, it was stored at -80°C for the next analysis (López-Rosero et al., in prep).

After synthesizing and preserving the cDNA at -80°C, three techniques were used for the identification of arbovirus: i) End-Point PCR, ii) Real Time PCR and iii) Sanger Sequencing.

End-Point PCR.

Flavivirus: A mix was prepared with 11.25 µl of nuclease-free water, 5 µl of buffer 5X green, 2 µl $MgCl_2$ mM, 0.5 µl of nucleotide mix 10 mM, 2.5 µl of primer forward (CFD2), 25 of primer µl reverse (MA) (Table 1), 0.25 µl GoTaq and 1 µl of sample. The amplification of the samples was configured in the thermo-cycler in the following way: i) 94°C for 2 mins, ii) 35 cycles of: 94°C for 45 seconds, 55°C for 1 min, and 72°C for 1 min, iii) 72°C for 7 mins (Kuno, 1998).

For Dengue, the same protocol was followed for the creation of the mixture, as well as the thermo-cycler settings that were mentioned in Flavivirus. The only difference was the primers. In Dengue, the primer used for forward was (D1-F), and for the reverse (D2-R) (Table 1) (Lanciotti et al., 1992).

However, for Zika, there were only a few changes in the protocol for the creation of the mixture because 8.34 µl of nuclease-free water, 3.8 µl of primer forward (ZIKVENVF), and 4.11 µl of primer reverse (ZIKVENVR) (Table 1) were added. Furthermore, there were changes in the thermo-cycler settings. These were: i) 95 °C for 2 mins, ii) 35 cycles of: 95°C for 20 seconds, 55°C for 20 seconds, 68°C for 30 seconds, iii) 68°C for 7 mins (Faye et al., 2008).

The samples for all three cases were revealed in 1.5% agarose gels with ethidium bromide as an intercalating agent.

Real Time PCR and Sanger Sequencing

For the detection of Zika, Dengue and Chikungunya multiplex, a bio*Perfectus* kit was used. For this procedure, the bio*Perfectus* procedure was followed, utilizing a Bio-Rad

detection machine in real time, being this one of the pieces of equipment that can be used for the acquired kit (bio*Perfectus* technologies, 2021).

Once the samples were analyzed with the RT-PCR kit, the Sanger sequencing was proposed and practiced with the goal of verifying data. 4 independent reactions were carried out, each one mixed with: the amplicon mixtures acquired during the End-Point PCR analysis for Dengue and Zika, primers for Dengue and Zika (Table 1), 4 2'-deoxynucleotides (dNTP) and 4 dideoxynucleotides (ddNTP), with one of these four in a lower proportion. Once the synthesis was obtained, the fragments were identified in a polyacrylamide gel (SEIMC, 2021).

Bioinformatic analysis

Once the results of the Sanger sequencing were obtained, the synthesized sequences were read with the Geneious Prime software. The starting point was the general assembly of Dengue and another general assembly of Zika. This allowed the distinction of which samples managed to align, which led to the cleansing of the sequences.

Once finalized, the National Center for Biotechnology Information's (NCBI) nucleotide tool BLAST was used for the identification of the assembled samples.

Results

The total amount of mosquitoes collected in the two Manabí communities was 468 specimens, grouped in 72 pools. Of those specimens, a total of 385 mosquitoes, 62 males and 323 females were collected in Caja Fuego. While 83 mosquitoes, 40 males and 43 females, were found in the San Gregorio community. The mosquito recollection was within the expected parameters in the rainy season.

In the end point PCR, 11 positive samples for Dengue and 7 for Zika were observed, however, the results of the RT-PCR did not detect any arboviruses. Of the 72 initial pools, only 2 Zika positive pools were obtained with Sanger sequencing (samples 21 and 53) (2.78%), corresponding to one within the Comunidad Caja Fuego and another for San Gregorio; it must be mentioned that both pools are from female samples of *A. aegypti*. The comparative results of the three techniques used for the identification of arbovirus in *A. aegypti* are shown in the following table (Table 2).

Discussion

The information about arboviruses is very important in Ecuador, as these are the causes of various high-impact pathologies within Ecuadorian society and may cause death to the hosts of these viruses. Due to the increasing health burden posed by endemic diseases transmitted by the Dengue, Zika, and Chikungunya viruses, it is important to implement new techniques which facilitate the detection and identification of these viruses; not only in humans, but simultaneously in their vectors, improving their control**.**

In this study, of the three techniques used, it is speculated that most sensitive is the RT-PCR multiplex (bio*Perfectus* technologies, 2021). This technique is extremely important due to the benefit of higher sensitivity and the amplification of multiple viruses at a time in comparison to the traditional method. However, in the case of this study, the quality of the samples was very poor and a possible degradation in RNA could be deduced. This could have happened due to i) the isolation with Trizol, ii) transportation time, iii) long time periods in which the sample was exposed to room temperatures (Hernández-Ruiz et al., 2019)**.**

The kit used for RT-PCR has limitations in its use, such as: i) negative results do not exclude the presence of Zika, Dengue and Chikungunya; therefore, it must not be considered as the only detection parameter, ii) results depend on collection of the mosquitoes, transportation, storage, and their later processing, iii) false negatives due to the presence of inhibitors, iv) false negatives as demonstrated through the Sanger sequencing results, and v) evolution of viruses and mutations (bio*Perfectus* technologies, 2021)**.**

In this study, the results obtained through Sanger sequencing (2 positive Zika samples) draw attention to the MSP report in the Manabí province. In 2023, there were a total of 99 Dengue cases reported within Manabí. However, there were no reported cases of Zika (MSP, 2023b). The analysis has made it evident that there are discrepancies when comparing the report with this study's results. This demonstrates that people within the collection zones where these mosquitoes were acquired find themselves at risk of infection due to the Zika virus.

Finally, in order to limit the degradation of RNA samples, modifying the extraction protocol is recommended to reduce the exposure times of the samples when they are handled (Liu & Harada, 2013)**.**

To obtain more information about these arboviruses and complement the studies of any of these techniques used (end point PCR, RT-PCR multiplex, and sanger sequencing) the following are recommended: i) purification of the samples, ii) increase in sample size, employment of CAP questionnaires, and iii) implementation of serology studies. These are vital to acquire a comprehensive overview of the aforementioned diseases, and all its protagonists.

In conclusion, it is sought to give a better concept, vision, and reduction in the time it takes to identify Dengue, Zika and Chikungunya in set populations without direct

intervention towards the humans, additionally reducing the risk of infection, alerting the communities, and implementing better vigilance campaigns in this way, together with MSP.

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442 **Tabla 1.** Primers used in the three methods to detect different arboviruses.

	Primer pair (code)	Amplicon	Source
Flavivirus	**MA:** 5'-CATGATGGGRAARAGRGARRAG-3' **CFD2:** 5'-GTGTCCCAGCCGGCGGTGTCATCAGC-3'	260 pb	(Kuno, 1998)
Dengue	**D1-F:** 5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' **D2-R:** 5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	511 pb	(Lanciotti et al., 1992)
Zika	**ZIKVENVF:** 5'-GCTGGDGCRGACACHGGRACT-3' **ZIKVENVR:** 5'-RTCYACYGCCATYTGGRCTG-3'	364 pb	(Faye et al., 2008)

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446 **Tabla 2.** Results of the different arboviruses found with the three applied techniques.

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	End-Point PCR	RT-PCR kit (bio*Perfectus*)	Sanger sequencing
Flavivirus	>75%	N/A	N/A
Dengue	11	0	0
Zika	7	0	2

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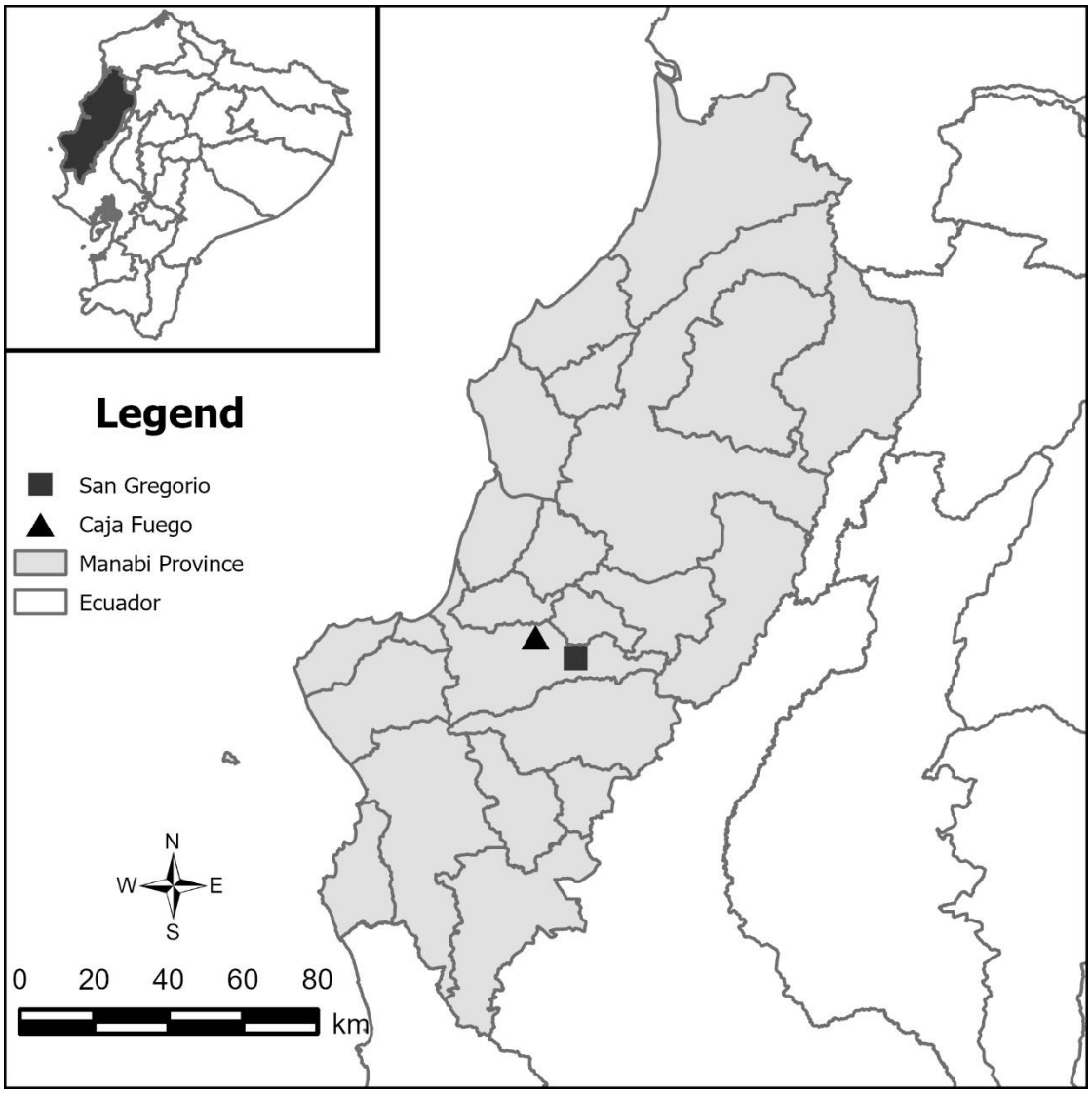
449 **Figure Legends**

450



451

452 **Figure 1.** Map of Manabí, Ecuador. A. Caja Fuego, rural community. B. Andrés Vera,

453 San Gregorio - marginal urban, both in Portoviejo County.

454

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6. Chemical analyses of component compounds must be included in the study.

Burgess ER, King BH, Geden CJ. 2020. Oral and topical insecticide response bioassays and associated statistical analyses used commonly in veterinary and medical entomology. J. Insect Sci. 20(6):1-9. https://doi.org/10.1093/jisesa/ieaa041
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[1]Aultman KS, Walker ED, Gifford F, et al. 2000. Managing risks of arthropod vector research. Science 288 (5475): 2321-2322. DOI: 10.1126/science.288.5475.2321

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Schmidt LS, Schmidt JO, Rao H, et al. 1995. Feeding preference and survival of young worker honey bees (hymenoptera: Apidae) fed rape, sesame, and sunflower pollen. J. Econ. Entomol. 88(6):1591-1595. https://doi.org/10.1093/jee/88.6.1591.

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White House. 2015a. National strategy to promote the health of honey bees and other pollinators. Pollinator Health Task Force. Available from https://obamawhitehouse.archives.gov/sites/default/files/microsites/ostp/Pollinator%20Health%20Strategy%202015.pdf

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Inventor(s), Assignee, Assignee name. Year. Title. Country Patent Number.
Pound M, Miller A, LeMeilleur A. 1994. Device and method for use as an aid in control of ticks and other ectoparasites on wildlife. U.S. Patent #5,367,983.

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[dataset]* Author surname(s) and initial(s), et al. Publication year. Title. (repository or archive name). Identifier
*The inclusion of the [dataset] tag at the beginning of the citation helps us to correctly identify and tag the citation. This tag will be removed from the citation published in the reference list.

Software citations should include the minimum information recommended by the FORCE11 Software Citation Implementation Group:

Author/developer surname(s) and initial(s), et al. Release date. Title. Publisher. (repository or archive name). Identifier
If there is an article describing the software, it is recommended to cite both the software and the article.

Tables

- Tables should be editable tables in a Word document.
- If a table continues on more than one page, repeat column headings on subsequent page(s).
- All columns must have headings.
- Leave no space between lowercase letters and their preceding values (e.g., 731.2ab).

- Do not footnote the title—use the unlettered first footnote to include general information necessary to understand the title (e.g., define terms, abbreviations, and statistical tests).
- Use approved abbreviations or abbreviations already defined in the text and define others in the general footnote.
- Use the following abbreviations in the body or column headings of tables only: amt (amount), avg (average), concn (concentration), diam (diameter), exp (experiment), ht (height), max (maximum), min (minimum), no. (number), prepn (preparation), temp (temperature), vs (versus), vol (volume), wt (weight) Jan (January), Feb (February), Mar (March), April, May, June, July, Aug (August), Sept (September), Oct (October), Nov (November), and Dec (December).

Figures

- Figures should be at least 300 dpi, or 1200 dpi for line graphs.
- The quality in which figures are submitted is the quality in which they will print—please ensure figures are high quality.
- The following file types of figures are accepted: tif (preferred), eps (preferred), rtf, ppt/pptx, pdf, ps, psd, ai, gif, png. Figures should be in their native format for best quality.
- Figures should be prepared in CMYK colour.
- The quality in which figures are submitted is the quality in which they will print—please ensure figures are high quality.
- Each figure should be submitted as an individual file. Please do not copy and paste them into the main document.
- Maximum height: 240 mm.
- Maximum width (one-column figure): 82 mm.
- Maximum width (two-column figure): 171 mm.
- For more information on preparing figures, see OUP's Author Resource Centre on figures.
- All authors are required to pay additional charges for colour figures. Authors may elect to publish in grayscale in print and in colour online for no charge. Authors electing to publish figures in colour online and in grayscale in the print edition must include grayscale images with their submission files. Please make sure your images are legible in grayscale and that image captions do not point to colored arrows, markers, or descriptors.

Graphical Abstracts

Authors are encouraged to submit a graphical abstract as part of the article, in addition to the text abstract. The graphical abstract should clearly summarize the focus and findings of the article, and will be published as part of the article online and in PDF. The graphical abstract should be submitted for peer review as a separate file, selecting the appropriate file-type designation in the journal's online submission system. The file should be clearly named, e.g. *graphical_abstract.tiff*. See this page for guidance on appropriate file format and resolution for graphics. Please ensure graphical abstracts are in landscape format.
Design tips and more information about graphical abstracts.
Note that graphical abstracts will be subject to any print reproduction charges that the journal levies for colour figures.

Supplementary Material

Supplementary Material may be submitted in the form of one or more files to accompany the online version of an article. Such material often consists of large tables, data sets, or videos that are not possible or convenient to present in print media. Supplementary Material represents substantive information to be posted on the ESA journal website that enhances and enriches the information presented in the main body of a paper; however, the paper must stand on its own without the need for the reader to access the supplementary information to understand and judge the merits of the paper. All Supplementary Material must be provided at the time of manuscript submission and will be distributed to reviewers as part of the normal peer-review process. It will not be edited during production and will be published as submitted.

Supplementary Material should be:

- Referenced in the body of the main paper (e.g., Supp. Table S1), where a link will take the online reader to the file.
- Be labeled with an appropriate title and caption.
- Citations for any literature referenced within a Supplementary Material file should be listed in a References Cited section at the end of the file, even when a citation is duplicated in the main body of the paper.
- Videos should be kept to a reasonable size to facilitate downloading by readers.

Videos and Animations

Videos should be submitted as .mp4 files, at the highest possible resolution, and include a still image to represent the video in the PDF.

Please do not submit animated gif files, which may be published as single image. Instead, please convert the gif to an .mp4 file.

If supplied alongside the manuscript and cited within the article text, published videos will appear as streamable content within the article body. We cannot use videos hosted on third-party sites such as YouTube, as the link may expire.

Species Authority, Order, Family, and Common Names

- Authors should provide the authority, order, and family for all organisms that are central to the paper (including plants, bacteria, and other non-arthropod organisms) at the first mention of the organism. It is the author's responsibility to provide accurate authority, order, and family information. Organisms mentioned in passing or whose importance to the paper is limited do not need to have full authority, order, and family listed, nor do mentions of common names of groups (e.g., mosquitoes, beetles, ticks, etc.).
- If a species name is included in the title of a paper, either the ESA-approved common name or the Latin name, the order and family should also be provided. If the order and family of an organism is provided in the title of the paper, it does not need to be provided again in the abstract and main text.
- If an organism is not listed in the title but is central to the paper, the order and family should be provided at first mention of the organism in both the abstract and the main text.
- The taxonomic authority of an organism that is central to the paper should be given the first time the organism is mentioned in both the abstract and the main text, but not in

the title. For tables that include lists of species, authority should be given for each species if it is the first time it is being mentioned in the paper.

- If multiple organisms in the same genus are central to the paper, order and family only need to be provided for the first species mentioned in the genus. If multiple organisms are central to the paper, are in different genera, but are in the same order and family, order and family should be provided at first mention of each organism.
- If two organisms that share the same order and family are mentioned in the title or listed in the text of the paper together, the order and family should be placed after the first species listed and does not need to be repeated after the second species.
- A genus can be abbreviated after the first mention (except to start a sentence, in which case the genus should be spelled out). If two species belong to different genera but the genera start with the same letter, the first two letters can be used for abbreviations.
- Only ESA approved common names should be used. Common names are lower case, except for proper nouns and their derivations.

Statistics

All data reported (except for descriptive biology) must be subjected to statistical analysis. Results of statistical tests may be presented in the text, in tables, and in figures. Statistical methods should be described in Materials and Methods with appropriate references. Descriptions should include information such as sample sizes and number of replications. Only t-tests, Chi square, and analyses of variance require no citation. Cite the computer program user's manual in the References Cited.

Probit/logit

When presenting results of probit/logit analysis, the following columns should be included in tables in the following order (left to right); n, slope + SE, LD (or LC) (95% CL), and chi-square. When a ratio of one LD versus another is given, it should be given with its 95% CI. Statistical tests to show what model best fits data intended to estimate the 99.9986% level of effectiveness should be presented to justify use of any model, including the probit model. Thus, we do not recommend use of the Probit 9 without tests to show that the probit model fits the data.

Analysis of Variance or t-test

When presenting the results of analysis of variance or a t-test, specify F (or t) values, degrees of freedom, and P values. This information should be placed in parentheses in the text. Example: ($F = 9.26$; $df = 4, 26$; $P < 0.001$). If readability of the text is affected by the presence of repeated parenthetical statistical statements, place them in a table instead.

Regression

In regressions, specify the model, define all variables, and provide estimates of variances for parameters and the residual mean-square error. Italicize variables in equations and text.

Variance and sample size

Include an estimate of the variance (or standard error) and sample size for each mean regardless of the method chosen for unplanned multiple comparisons. The use of

Duncan's Multiple Range Test (DMRT) is not acceptable as a mean separation test as it was designed to be a very liberal test intended to find even minor differences in resistance between plant lines used for breeding.

Model Analysis, Guidelines, Equations, and Computer Code

Model Analysis

At the beginning of the manuscript, authors should state clearly the goals of their model construction and analysis. Evaluation by reviewers depends upon these goals and the type of model. Authors should attempt to describe the main conclusions, limitations, and sensitivity of results to assumptions. For stochastic models, describe the variability in the results.

Modeling Guidelines

The following guidelines pertain to any mathematical model calculated for purposes other than statistical analysis.

- Authors must adequately describe both model structure and model analysis.
- Authors must explain and justify original equations and computer programs or justify the selection of a published software package used in the computation of models.
- Model structure and steps in the analysis must be described in the Materials and Methods section.
- Without presenting extensive computer code, the text must permit an understanding of the model that would allow most mathematically inclined scientists to duplicate the work.
- Present all equations that represent the biology of the system being modeled.
- Unless their derivation is self-evident, show how the equations were derived and mention the underlying assumptions.
- Express how the equations are solved over time and space.
- Provide references for standard techniques (e.g., matrix manipulation, integration).
- Define all variables and parameters in each equation and describe their units (e.g., time, space, and mass).
- In the Materials and Methods or Results section, present the range of parameter values included in the model, and describe the uncertainty in or range of validity of these values.

Equations

Consult Mathematics into Type for correct formatting of equations and mathematical variables. Italicize all mathematical variables.

Validation or Testing of Model Results

Data used for validation must be independent of data used to build or calibrate the model. Authors must state why the model did not require testing (e.g., theoretical study), why it cannot be tested (e.g., lack of data), or how it was tested.

Structure of Computer Code

For models solved or simulated by computers, mention the programming language and computer used. Describe the important numerical methods used in calculating the model (e.g., integration and random number generation). Mention how the program's logic and

algorithms were tested and verified. When published software is computed, provide a reference and state which procedures were used. Discuss in any section of the manuscript the limitations of the published software. Original computer programs should be made available at the request of reviewers and readers.

Taxonomic Papers

ICZN compliance

JIS is compliant with the International Code of Zoological Nomenclature, and the publishing processes of the journal ensure that new acts of nomenclature are considered valid under the code. JME is permanently archived with CLOCKSS, LOCKSS, and Portico in a manner that preserves the content and layout of the work.

Nomenclatural Works and Zoobank Registration

You will be asked during the submission process whether your article contains a new nomenclatural act. If it does, in order to comply with ICZN regulations, the author will need to register the article in ZooBank and insert a nomenclatural statement, which includes a Life Science Identifier (LSID), into the article. The registration can be made at first revision of the article. Instructions for this process can be found here. When published, your article will also include the online publication date, and the statement "Version of Record, first published online [online publication date], with fixed content and layout in compliance with Art. 8.1.3.2 ICZN." Following publication, the editorial office will update your ZooBank entry with the DOI, Volume, and Issue information. When you have registered the paper, please send the article tracking number and LSID to the editorial office (pubs@entsoc.org) to record in the peer-review system.

Taxonomic Style

Follow the *International Code of Zoological Nomenclature* for taxonomic style. Some specific areas to note:

- Center the heading that indicates the name of the taxon in bold type.
- Center figure numbers in parentheses under the main heading; do not use bold type.
- Start all synonomies at the left margin with runovers indented.
- Include authors and date.
- References must appear in References Cited section.
- Use telegraphic style throughout descriptions.

Taxonomy Headings

Use only acceptable 3rd-level subheadings such as:

- Male
- Female
- Material Examined
- Type Material
- Distribution
- Etymology
- Biology
- Discussion

Avoid using "Description" as a subheading.

Dates

- Use Roman numerals I through XII to designate month of collection.
- Use Arabic numerals 0000 through 9999 to designate collection years. Do not abbreviate other years, including the 21st century, except when explicitly transcribing exactly what appears on specimen labels.
- Express dates in this format: day-month (use a Roman numeral)-year. Example: 2-V-97.

Locality Other than Principal Types

- Start with the largest area followed by successively smaller areas separated by colons.
- Capitalize countries.
- Arrange data for each locality in the following order: count of specimens and sex or stage (as applicable), city or vicinity, date, collector, and depository. Example: MEXICO: Tamaulipas: 1 male, 1 female, Ciudad Mante, 15-III-97, K. Haack; 5 females, Ciudad Victoria, 3-VII-99, C. Hughes, MCZ.
- Arrange localities alphabetically.
- Use a semicolon to separate data for different localities.
- Define depositories in the Materials and Methods.

Type Material

Start description with the principal type in capital letters. Follow this immediately with count and sex of specimens (use male and female symbols if possible), then place additional data in the order of locality, date, additional data, and collector. Separate these items with commas. Example: HOLOTYPE: 1 male, Locust Grove, VA, 22-X-98, on Cercis canadensis, R. H. Foote. PARATYPES: 2 males, same data.

Voucher Specimens

Voucher specimens of arthropods serve as future reference for published names used in scientific publications. Authors are required to deposit voucher specimens in an established, permanent collection and to note in the published article that the expected deposition has been made, its location, and the collection accession number. Authors should contact the curator of a voucher repository before deposition concerning the procedures required for curation to ensure that the collection will accept the voucher materials. The designation and proper labeling of voucher specimens is the author's responsibility. When available, at least three specimens should be deposited. Each specimen should have the following information provided at the time of deposition:

- Standard label data that are required for the specimens collection (i.e., locality, date of collection, collector, host, ecological data, whether the specimen is from a laboratory collection, etc.).
- An identification label that includes the identifier and date of identification.
- A label that designates the specimen as "voucher."

Immutable Advance Access

JME publishes articles online ahead of inclusion in an issue via OUP’s Advance Access.

In order to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN) with regard to nomenclatural works, articles published by ESA's journals that include nomenclatural information will be immutable. Additionally, no changes will be allowed to any article without the publication of a correction clearly stating the changes that have been made. Therefore, it is the responsibility of the authors to carefully check their proofs for accuracy, and to notify the publisher of any changes that are necessary prior to Advance Access publication.

Gene Sequencing

- Inclusion of a GenBank/EMBL accession number for primary nucleotide and amino acid sequence data is required.
- Sequences from new species and new genes must indicate the proportion of the gene sequenced and should include data from both strands.
- The accession number may be included in the original manuscript or the sequence may be provided for review and an accession number provided when the manuscript is revised.

Availability of Data and Materials

Where ethically feasible, *JME* strongly encourages authors to make all data and software code on which the conclusions of the paper rely available to readers. We suggest that data be presented in the main manuscript or additional supporting files, or deposited in a public repository whenever possible. Information on general repositories for all data types, and a list of recommended repositories by subject area, is available here.
ESA journals are integrated with Dryad (www.datadryad.org), an online data repository. Upon submission, authors will be sent a link to easily deposit the full dataset(s) behind their research in Dryad. Datasets deposited in Dryad are given unique DOIs, linked to the corresponding paper, and are available to be reviewed by subject editors of the journal upon request. Depositing data in Dryad is optional.
If you would like your paper to link to the dataset, data should be deposited prior to acceptance. Authors are required to pay Dryad for depositing data, but this fee is only charged if a paper is accepted.

ORCID

JME requires submitting authors to provide an ORCID iD at submission to the journal. More information on ORCID and the benefits of using an ORCID iD is available. If you do not already have an ORCID iD, you can register for free via the ORCID website. Coauthors are encouraged to link their ORCID iDs to their online review system accounts. Because the coauthor must login to the ORCID system to verify their identity, this process cannot be completed by the corresponding author or staff.

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the authors are required to update the status of any preprint, including your published paper's DOI, as described on our Author Self-Archiving policy page.

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In order to comply with the requirements of the International Commission on Zoological Nomenclature (ICZN) with regard to nomenclatural works, ALL articles published by ESA's journals, regardless of whether they include nomenclatural information, will be immutable; this means that no changes will be allowed to any article without the publication of an erratum clearly stating the changes that have been made. Therefore, it is the responsibility of the authors to carefully check their proofs for accuracy, and to notify the publisher of any changes that are necessary prior to Advance Access publication.

Revised Papers

For revised papers, we have a few more formatting requirements than we do for new submissions, to ensure the paper is ready for publication if it is accepted.
Response to reviews

Provide a document listing how you changed your manuscript in response to each point mentioned by the reviewers. If you did not implement a change suggested by a reviewer, please explain why.

Style

For any matters of editorial style not covered in these author guidelines, ESA journals adhere to the Council of Science Editors (CSE) Style Guide.
Measurements

Use metric units. English units may follow within parentheses if needed.

Nomenclature

- Give full scientific name and authority at first mention of each organism (including plants and non-insect animals) in the abstract and again in the text.
- Only use common names listed in the current ESA Common Names of Insects & Related Organisms online database. Common names should be lowercase, except for proper nouns and their derivations.
- When possible, please provide as much genetic and/or colony information available is useful (for example, "Rockefeller colony of Aedes aegypti (L.)"). Including geographic origin and generations in culture is also useful, but may not always be known and is not required.

Submissions of Cover Photographs

Authors are encouraged to submit insect photos for the cover of the journal. Photos must be of living (not pinned) insects and must be submitted as high-resolution (300 dpi or higher) image files. If you wish to crop your image to the exact dimensions of our cover, the dimensions to use are W: 222 mm x H: 203 mm; however, photos that do not

match those dimensions can still be accepted. The insect species featured on the cover of a given issue must be mentioned in the issue in question.
Photographers will be asked to give ESA one-time permission to use the photo on the journal cover and will retain their copyright of the photo.
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