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Acetylcholinesterase activity and micronucleated cells of *Myotis velifer* in agricultural landscapes

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Bats use agricultural landscapes as foraging areas and may be exposed to pesticides through the consumption of contaminated prey. Biomarkers can detect sublethal responses associated with such exposure; among them, acetylcholinesterase (AChE) activity reflects neurotoxic alterations, while micronucleated cells in the buccal mucosa indicate cytogenetic damage. Given this context, it is important to evaluate the biological responses of *M. velifer* in agricultural environments by considering both biomarkers and landscape characteristics. Therefore, our objective was to evaluate plasma acetylcholinesterase activity and the frequency of buccal micronucleated cells in *M. velifer* individuals from locations with various agricultural contexts in central Mexico. We captured 31 adult males in Tlaxco, Tlaxcala (n = 8), Nauzontla, Puebla (n = 13), and Aquixtla, Puebla (n = 10). At each site, the landscape within circular areas of 10 km in radius around each roost was described using land-use cartography to quantify the main land-cover types. AChE activity was determined using a colorimetric method, and the frequency of micronucleated cells was assessed in Giemsa-stained buccal mucosa smears. Agriculture was the dominant land-cover type in Tlaxco and Nauzontla, whereas primary vegetation predominated in Aquixtla. AChE activity differed among localities, being higher in Tlaxco than in Nauzontla and Aquixtla. The frequency of micronucleated cells also varied, being higher in Tlaxco than in Nauzontla. Although Tlaxco and Nauzontla had similar proportions of agricultural land, the biological responses of local bats differed between these two localities. These results show that both biomarkers provide complementary information and allow the identification of distinct biological responses across agricultural landscapes with different production contexts. Overall, this study demonstrates the usefulness of integrating landscape descriptions with enzymatic and cytogenetic biomarkers to assess sublethal responses in insectivorous bats.

Keywords: biomarkers, ecotoxicology, genotoxicity, neurotoxicity, pesticides.

Los murciélagos utilizan los paisajes agrícolas como áreas de forrajeo y pueden estar expuestos a plaguicidas mediante el consumo de presas contaminadas. Los biomarcadores permiten detectar respuestas subletales asociadas con dicha exposición; entre ellos, la actividad de la acetilcolinesterasa (AChE) refleja alteraciones neurotóxicas, mientras que la presencia de células micronucleadas en la mucosa bucal indica daño citogenético. En este contexto, es importante evaluar las respuestas biológicas de *M. velifer* en ambientes agrícolas, considerando tanto los biomarcadores como las características del paisaje. Por lo tanto, nuestro objetivo fue evaluar la actividad de la acetilcolinesterasa plasmática y la frecuencia de células micronucleadas en la mucosa bucal de individuos de *M. velifer* provenientes de localidades con diferentes contextos agrícolas en el centro de México. Capturamos 31 machos adultos en Tlaxco, Tlaxcala (n = 8), Nauzontla, Puebla (n = 13) y Aquixtla, Puebla (n = 10). En cada sitio, se caracterizó el paisaje dentro de áreas circulares de 10 km de radio alrededor de cada refugio, utilizando cartografía de uso de suelo para cuantificar los principales tipos de cobertura del suelo. La actividad de AChE se determinó mediante un método colorimétrico, y la frecuencia de células micronucleadas se evaluó en frotis de mucosa bucal teñidos con Giemsa. La agricultura fue el tipo de cobertura del suelo dominante en Tlaxco y Nauzontla, mientras que la vegetación primaria predominó en Aquixtla. La actividad de AChE difirió entre localidades, siendo mayor en Tlaxco que en Nauzontla y Aquixtla. La frecuencia de células micronucleadas también varió, siendo mayor en Tlaxco que en Nauzontla. Aunque Tlaxco y Nauzontla presentaron proporciones similares de superficie agrícola, las respuestas biológicas de los murciélagos locales difirieron entre ambas localidades. Estos resultados muestran que ambos biomarcadores proporcionan información complementaria y permiten identificar distintas respuestas biológicas en paisajes agrícolas con diferentes contextos productivos. En conjunto, este estudio demuestra la utilidad de integrar la caracterización del paisaje con biomarcadores enzimáticos y citogenéticos para evaluar respuestas subletales en murciélagos insectívoros.

Palabras clave: biomarcadores, ecotoxicología, genotoxicidad, neurotoxicidad, plaguicidas.

Insectivorous bats frequently use agricultural landscapes as feeding grounds, where they contribute to the natural control of insect pests associated with crops (Kunz *et al.* 2011). This interaction can expose them to pesticides, primarily through consumption of contaminated prey and, to a lesser extent, through contact with treated surfaces or through the dispersion of pesticides during their application (Köhler and Triebskorn 2013; Bayat *et al.* 2014).

Exposure to pesticides can cause direct mortality and sublethal responses that alter neurological, physiological, or genetic processes without immediate external signs (Fenech 2000; Chambers and Oppenheimer 2004; Thomas *et al.* 2009; López-Durán *et al.* 2018). These factors are relevant to wildlife in general and are particularly important for bats, given their ability to move and their use of different areas during foraging. These characteristics can favor repeated exposures and make it difficult to identify their effects under natural conditions (O'Shea and Johnston 2009; Stahlschmidt *et al.* 2017).

Biomarkers enable detection of biological changes associated with pollutant exposure or effects before alterations are evident at higher organizational scales (van der Oost *et al.* 2003; Walker *et al.* 2005). Among them, acetylcholinesterase (AchE) activity is used to evaluate responses related to organophosphate pesticides and carbamates, both of which can inhibit this enzyme and affect nerve transmission (Clark and Rattner 1987; van der Oost *et al.* 2003; Chambers and Oppenheimer 2004). On the other hand, micronuclei derive from chromosomal fragments or whole chromosomes that are not incorporated into the main nucleus during cell division, so their frequency is an indicator of cytogenetic damage accumulated during the renewal of the tissue analyzed (Fenech 2000; Thomas *et al.* 2009). Consequently, both biomarkers provide complementary information: AChE reflects an enzymatic response primarily associated with neurotoxicity, while micronucleated cells indicate chromosomal alterations induced by genotoxic agents. Studies conducted in bats under free-living and experimental conditions have supported the sensitivity of these biomarkers, while also showing that their interpretation must consider the species, sample type, and the environmental context of the individuals evaluated (Benvindo-Souz *et al.* 2019; Sandoval-Herrera *et al.* 2021).

In this context, the cave myotis *Myotis velifer,* is a relevant species to evaluate biological responses in agricultural landscapes due to its insectivorous diet, wide distribution, and association with altered environments (Wilson 2007; Jones *et al.* 2009). It is a troglophile distributed from the south-central United States to Central America; it is widespread in Mexico, where it thrives in temperate environments at different altitudes (Fitch *et al.* 1981). In central Mexico, *M. velifer* makes seasonal movements between roosts associated with hibernation, mating, and raising young (Arratia 2000). In addition, it is directly exposed to environmental pollutants: organochlorine and organophosphate pesticides have been detected in guano, as well as organochlorine compounds and heavy metals in tissues, in areas of Texas; likewise, the seasonal activity of these colonies has been related to periods of greater agricultural activity (Land *et al.* 2019). Despite this background, information on the biological responses of *M. velifer* in the agricultural landscapes of central Mexico remains limited.

Given this scenario, information on the biological responses of *M. velifer* in agricultural environments is relevant for evaluating biomarkers that represent different physiological processes and for analyzing their variation across localities. Therefore, the objective of this study was to evaluate the variation in plasma acetylcholinesterase activity and the frequency of buccal micronucleated cells in *M. velifer* individuals from three localities with various land uses in central Mexico. To this end, the composition of land use around the bat roosts was characterized, and the two biomarkers were compared across localities. Our hypothesis is that differences in the agricultural context between localities would be reflected in the evaluated biomarkers, such that bats foraging in areas with greater agricultural activity would have lower plasma AChE activity and a higher frequency of buccal micronucleated cells.

Materials and methods

Study area. The study was conducted in three locations with known *Myotis velifer* roosts in central Mexico (Figure 1): El Túnel, in Cerro Huilapitzo, Tlaxco, Tlaxcala; Hacienda de Almeya, in Aquixtla, Puebla, and the Chicomoztoc cave in Nauzontla, Puebla.

El Túnel is located in Tlaxco, Tlaxcala (19°37'14" N, 98°02' 02" W; 3220 m.a.s.l.), in an area with a temperate sub-humid climate (Cw), temperatures between 6.5 °C and 22 °C, and vegetation dominated by pine-fir forest (García 2004; INEGI 2013). Hacienda de Almeya is located in Aquixtla, Puebla (19°44'16" N, 97°54'47.5" W; 2387 m.a.s.l.), in a region with a temperate sub-humid climate with summer precipitation (Cw), temperatures between 12 °C and 18 °C, and pine-oak vegetation (Rzedowski 1978; García 2004; INEGI 2013). The Chicomoztoc cave is located in Nauzontla, Puebla (19°57'54" N, 97°36'09" W; 1420 m.a.s.l.), in an area with a humid semi-warm climate (Ac), precipitation throughout the year, mean annual temperature close to 20 °C, and secondary vegetation derived from cloud forest (García 2004; INEGI 2013).

Each roost was sampled only once during 2025: Tlaxco in February, Nauzontla in May, and Aquixtla in July. The timing of sampling was defined based on the seasonal displacements described for *M. velifer* and the use of these roosts for hibernation, mating, and raising young (Arratia 2000). Consequently, the timing of sampling must be considered when interpreting observed differences across localities.

Land use characterization. The composition of the landscape around each roost was characterized using the Vector Dataset of Land Use and Vegetation, Series VII, at a scale of 1:250 000, from the National Institute of Statistics and Geography (INEGI 2021). The cartographic information was processed in QGIS version 3.40.0.

Circular zones of influence with a radius of ten kilometers were established around each roost to describe the composition of land uses in the environment where bats forage. This radius was used as a reference spatial scale

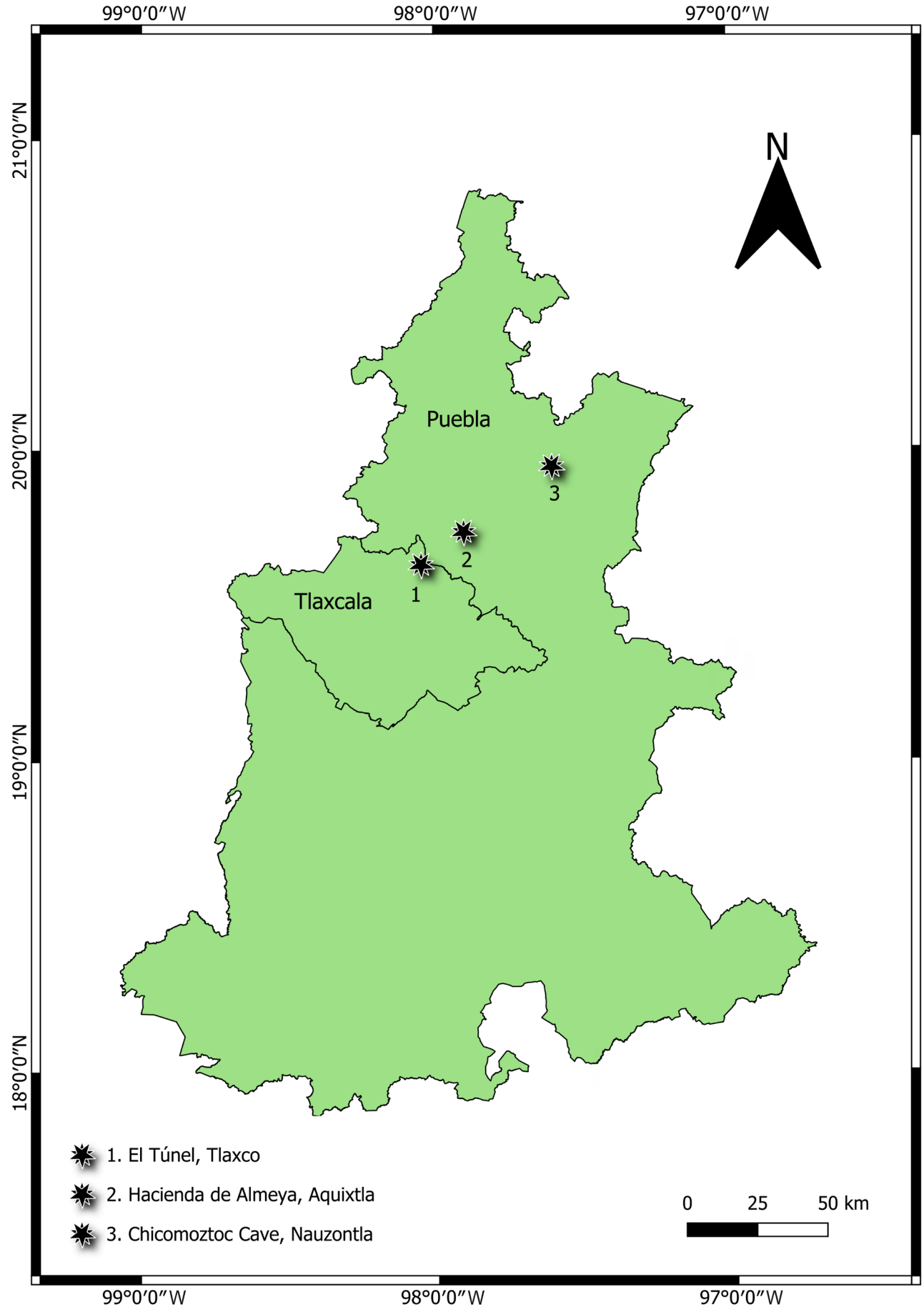

Figure 1. Map of the study sites: Chicomoztoc Cave and Hacienda de Almeya (Sierra Norte de Puebla, Puebla), and El Túnel (Sierra de Tlaxco, Tlaxcala).

and does not represent the maximum displacement range of *M. velifer*, for which daily foraging movements across greater distances have been documented (Hayward 1970). Within each zone of influence, the area corresponding to each land use and vegetation cover was quantified.

The original classes considered in the cartography were reclassified into four general categories: agriculture, primary vegetation, secondary vegetation, and human settlements (Thaden *et al.* 2023). The surface area of each category was calculated from polygon geometry, expressed as km^2 and as a percentage of the total area of each zone of influence. For surface calculations, the spatial layers were projected into the WGS 84/UTM zone 14N coordinate system (EPSG:32614), which enabled estimates in metric units.

Qualitative characterization of local phytosanitary management. The spatial characterization of land use was complemented by exploratory surveys of phytosanitary management practices at the study locations. These surveys were conducted in 2025 during field trips in Tlaxco (February), Nauzontla (May), and Aquixtla (July). Agricultural farmers, local workers, and distributors of crop supplies were asked open questions on major crops, pests of local importance, and products used for pest control. This information was recorded in field notes and was used for descriptive purposes to contextualize productive practices and exposure scenarios in each locality.

Capture and handling of bat specimens. In each locality, bats were caught from sunset over approximately five hours. Specimens were captured using a blow net, a mist net, or a harp trap, depending on the characteristics of each roost and the condition of the bats. Of the specimens captured, only 31 adult males of *M. velifer* distributed across the three study locations were included in the study: Tlaxco ($n = 8$), Nauzontla ($n = 13$), and Aquixtla ($n = 10$).

Taxonomic identification was carried out using specialized keys (Medellín *et al.* 2008; Álvarez-Castañeda *et al.* 2017). Sex was determined by observation of the external genitalia, and the adult stage was established from the degree of ossification of the metacarpal-phalangeal epiphyses (Kunz and Anthony 1982; Anthony 1988). Only adult males were included to avoid potential variability associated with sex and age.

Specimen capture, handling, and collection were performed in accordance with appropriate ethical guidelines accepted by Wilson *et al.* (1996) and following the recommendations of the American Society of Mammalogists for the use of wild mammals in research (Sikes *et al.* 2016). These activities were carried out under the scientific license SPARN/DGVS/13548/24, granted by the General Direction of Wildlife of the Board of Environment and Natural Resources of Mexico (SEMARNAT).

Sample collection and processing. Epithelial cells were obtained from the buccal mucosa by rubbing cotton swabs on the tongue, inner surface of the cheeks, and palate. The collected material was smeared on slides, fixed with 100% methanol for 15 minutes, and left to air-dry for subsequent staining and cytogenetic analyses (Tolbert *et al.* 1992; Muñoz *et al.* 2016).

Blood samples were obtained using two procedures. In individuals that were subsequently released, a maximum of 100 µL of blood was collected by cardiac puncture. After the procedure, individuals were hydrated with purified water and remained resting and under observation in cloth bags for several hours before being released at the capture site. In the nine individuals euthanized for organ extraction, blood samples were collected from the cervical region immediately after decapitation, obtaining up to 150 µL of blood. These samples were collected in heparinized tubes and centrifuged at 3000 rpm for 3 minutes to separate the plasma, which was then recovered and stored at −20 °C until the acetylcholinesterase activity test (Muñoz *et al.* 2016). Euthanasia procedures were performed following the recommendations of the American Veterinary Medical Association (AVMA 2020) and the American Society of Mammalogists (Sikes *et al.* 2016).

Acetylcholinesterase test. Plasma AChE activity was determined with an adaptation of the colorimetric method of Ellman *et al.* (1961), based on acetylthiocholine hydrolysis by acetylcholinesterase followed by the reaction of the thiocholine released with 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). This reaction produces the anion 5-thio-2-nitrobenzoate (TNB), which is then quantified spectrophotometrically. Although AChE is predominantly associated with erythrocytes, Ellman's method has adaptations for testing cholinesterase activity in different biological matrices, including plasma (Bernal-Hernández *et al.* 2018). In this study, the plasma-adapted method was used to determine AChE activity in this blood fraction. Twenty-five microliters of plasma, 25 µL of acetylthicholine, and 225 µL of SIGMA-ALDRICH DTNB were poured into each microplate well. The reaction was performed in the presence of SIGMA-ALDRICH tetraisopropyl pyrophosphoramide (ISO-OMPA), a selective butyrylcholinesterase inhibitor, to reduce the contribution of this enzyme in the estimation of AChE activity.

Plasma samples were tested in triplicate using a 1:100 dilution, and a reaction blank was included in which plasma was replaced by distilled water. Absorbance was recorded at 405 nm at room temperature for 15 minutes in a ChroMate microplate reader. Enzyme activity was calculated from the change in absorbance per minute in the linear phase of the reaction, using the respective molar extinction coefficient, and expressed as plasma U/mL. Since total protein concentration was not determined, the results were reported as plasma AChE activity rather than as specific activity.

Micronucleus assay. Epithelial cell smears from the buccal mucosa were stained with HYCEL 10% Giemsa for 25 minutes, rinsed under running water, and mounted with Entellan. One-hundred cells per individual were

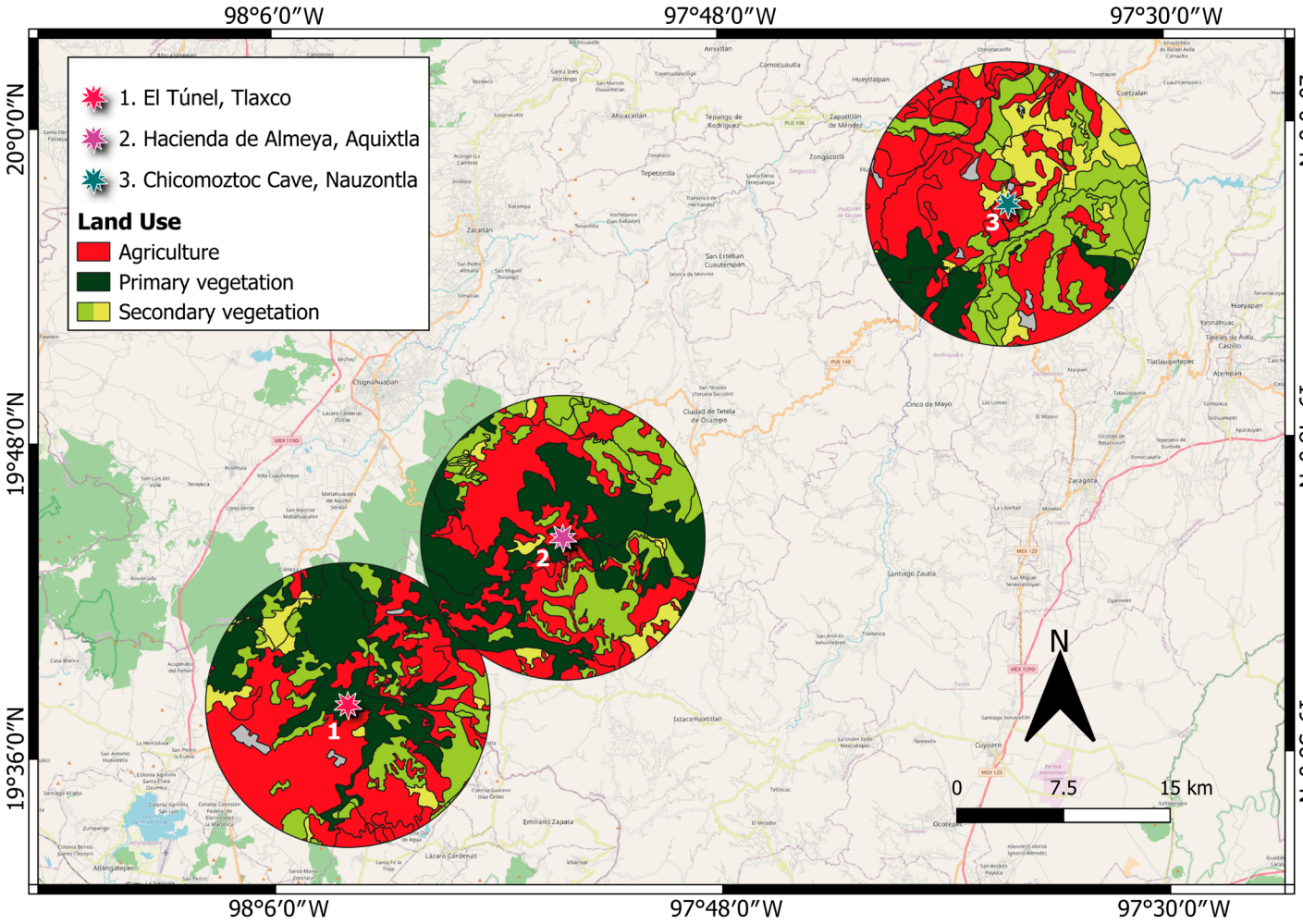

Figure 2. Land use and vegetation composition within 10-km buffer zones around *Myotis velifer* roosts in Tlaxco, Aquixtla, and Nauzontla. Land-cover classes were reclassified into agriculture, primary vegetation, secondary vegetation, and human settlements based on the Land Use and Vegetation Vector Dataset, Series VII, at a scale of 1:250,000 (INEGI 2021).

examined under light microscopy at 400×. Micronuclei were identified according to the criteria of Tolbert *et al.* (1992), that is, spherical or oval structures with a diameter between 1/3 and 1/16 of the main nucleus and a staining intensity similar to that of the latter. Each cell with one or more micronuclei was recorded as a micronucleated cell. The results were expressed as the frequency of micronucleated cells per 100 cells evaluated.

Statistical analysis. For plasma AChE activity and micronucleated cell frequency, descriptive statistics were calculated for each locality. Data were tested for normality using the Shapiro–Wilk test and for homoscedasticity using the Levene test.

Differences in plasma AChE activity between localities were analyzed using a one-way analysis of variance (ANOVA), followed by Tukey's post hoc test. As the frequency of micronucleated cells did not meet the normality and homoscedasticity assumptions, differences between localities were evaluated using the Kruskal–Wallis test, followed by Dunn's multiple comparisons. These tests were performed on GraphPad Prism 8 and $\alpha = 0.05$ was used to define statistical significance.

Results

Land uses in study sites. The composition of land use differed across study sites (Table 1; Figure 2). Crops were the predominant cover in Nauzontla (47.27%) and Tlaxco (46.55%), while they represented a lower proportion in Aquixtla (35.55%). Primary vegetation cover was highest in Aquixtla (35.80%), followed by Tlaxco (30.85%) and Nauzontla (10.05%). In contrast, secondary vegetation reached its highest proportion in Nauzontla (41.00%), followed by Aquixtla (28.65%) and Tlaxco (21.50%). Human settlements accounted for a low proportion of land use in Nauzontla (1.68%) and Tlaxco (1.10%) and were not recorded in the area studied in Aquixtla.

Table 1. Percentage composition of land-use and vegetation categories within a 10-km radius around *Myotis velifer* roosts.

Land use	Tlaxco (%)	Aquixtla (%)	Nauzontla (%)
Agriculture	46.55	35.55	47.27
Primary vegetation	30.85	35.80	10.05
Secondary vegetation	21.50	28.65	41
Human settlements	1.10	0	1.68

Acetylcholinesterase (AChE) activity. Acetylcholinesterase (AChE) activity in plasma showed significant differences

among study sites (ANOVA: $F2,28 = 6.731$, $p = 0.0041$; Figure 3). The values obtained were 0.091 ± 0.037 U/mL in Tlaxco ($n = 8$), 0.050 ± 0.01 U/mL in Nauzontla ($n = 13$), and 0.037 ± 0.03 U/mL in Aquixtla ($n = 10$). Tukey's post hoc test showed that the individuals captured in Tlaxco had significantly higher AChE activity than those in Aquixtla ($p = 0.0039$) and Nauzontla ($p = 0.0219$), while no differences were detected between Aquixtla and Nauzontla ($p = 0.6056$, considering $\alpha = 0.05$).

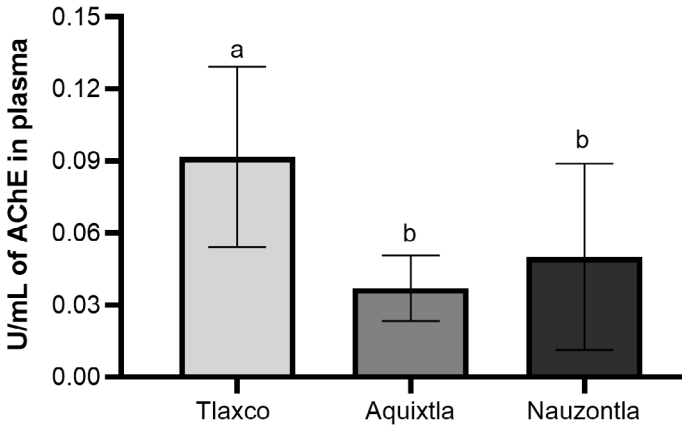

Figure 3. Plasma acetylcholinesterase (AChE) activity in *Myotis velifer* from Tlaxco (n = 8), Aquixtla ($n = 10$), and Nauzontla ($n = 13$). Values are expressed as mean ± SD. Significant differences among localities were observed (one-way ANOVA: $F_{2,28} = 6.731$, $P = 0.0041$). Different letters indicate significant differences among groups according to Tukey's test ($P < 0.05$).

Micronuclei. The values of micronucleated cells in the buccal mucosa were 1.25 ± 1.2% in Tlaxco, 0.30 ± 0.48% in Aquixtla, and (0.23 ± 0.8%) in Nauzontla. The Kruskal–Wallis test showed significant differences between localities ($H = 9.518$, $p = 0.0086$). Dunn's multiple comparison test indicated that individuals captured in Tlaxco had a significantly higher percentage of micronucleated cells than those from Nauzontla ($p = 0.0067$). No significant differences were detected between Tlaxco and Aquixtla ($p = 0.1006$), nor between Aquixtla and Nauzontla ($p = 0.9999$; $\alpha = 0.05$) (Figure 4).

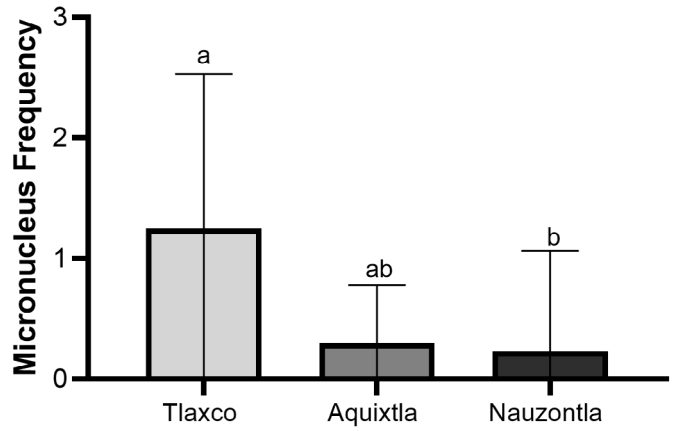

Figure 4. Frequency of micronucleated cells in the buccal mucosa of *Myotis velifer* from Tlaxco ($n = 8$), Aquixtla ($n = 10$), and Nauzontla ($n = 13$). Values are expressed as $\bar{x}$ ± SD. Significant differences among localities were observed (Kruskal–Wallis test: $H = 9.518$, $P = 0.0086$). Dunn's multiple comparisons test showed significant differences between Tlaxco and Nauzontla ($P = 0.0067$). Different letters indicate significant differences among groups ($P < 0.05$).

Discussion

Biomarkers are useful tools for detecting biological responses associated with pollutant exposure. In this study, the integration of plasma AChE activity, micronucleated cell frequency, and land-use characterization enabled comparison of individuals of *Myotis velifer* from three localities with different land-use compositions. Plasma AChE activity was significantly higher in bats from Tlaxco than in those from Aquixtla and Nauzontla, while the frequency of micronucleated cells was significantly higher in specimens from Tlaxco compared to those thriving in Nauzontla. These results indicate that both biomarkers reflect different biological processes and time scales. AChE reflects an enzymatic response that is inhibited by some insecticides, particularly organophosphates and carbamates, while micronucleated cells indicate chromosomal damage that can be caused by genotoxic agents, including some pesticides. Therefore, the higher frequency of micronucleated cells in Tlaxco is compatible with greater cytogenetic damage, while the high AChE activity recorded in bats from this locality does not correspond to the inhibition pattern expected in relation to more recent exposure to anticholinesterase compounds.

Plasma AChE activity. Plasma AChE activity differed significantly among the evaluated localities. Bats captured in Tlaxco had the highest enzymatic activity (0.091 U/mL), followed by those from Nauzontla (0.050 U/mL) and Aquixtla (0.037 U/mL). Although these results show clear differences between localities, the trend in this response differs from the expected pattern associated with recent exposure to organophosphate pesticides or carbamines, as these compounds usually inhibit AChE activity (Clark 1986; Fulton and Key 2001; Chambers and Oppenheimer 2004).

Our findings in Tlaxco contrast with the response of bats exposed to cholinesterase-inhibitor pesticides, in which a decrease in enzyme activity is generally observed. For example, Clark (1986) reported reduced brain cholinesterase activity in *Myotis lucifugus* exposed to methyl parathion, while Eidels *et al.* (2016) observed decreased plasma and brain cholinesterase activity in *Eptesicus fuscus* exposed to chlorpyrifos. Similarly, Sandoval-Herrera *et al.* (2023) recorded lower plasma AChE activity in wild populations of *Pteronotus mexicanus* associated with greater crop development, in addition to confirming this pattern in *Eptesicus fuscus* experimentally exposed to chlorpyrifos. Together, these studies show that inhibition of cholinesterase activity is the most common response to this type of compound, which highlights that the AChE activity observed in bats from Tlaxco does not follow this expected pattern.

The quantitative comparison between the values obtained in this study and those previously reported must be interpreted considering the differences among the species evaluated, the specific experimental or field conditions, the type of sample analyzed, the substrate used, the selectivity of the assay, and the units used

to report enzymatic activity. In the present work, AChE activity was determined in plasma and in the presence of ISO-OMPA to reduce the contribution of butyrylcholinesterase, while Clark (1986) and Eidels *et al.* (2016) evaluated cholinesterase activity in different tissues and under experimental exposure conditions. Therefore, the most robust comparison is found in the trend in the response and the differences across localities, rather than in the direct equivalence of absolute values.

The observed differences in AChE activity may be related to the physiological state of the individuals and the environmental conditions during sampling. Cholinesterase activity can be modified by temperature, seasonality, body size, and food availability (Durieux *et al.* 2011; Suchiang *et al.* 2011; Pala and Serdar 2018; Bernal-Rey *et al.* 2020). This explanation is particularly relevant because the localities were sampled in different months and at different stages of the annual cycle of *M. velifer*. The individuals from Tlaxco were captured in February, at the end of the hibernation period; those from Nauzontla, in May, during the reproductive season; and those from Aquixtla, in July, when this roost serves as a transit site (Arratia 2000). Therefore, differences in AChE activity could be associated with the physiological and environmental changes typical of these seasonal stages.

In addition to this explanation, the lower AChE values recorded in bats from Aquixtla and Nauzontla are consistent with a possible recent exposure to cholinesterase-inhibitor compounds, given the history described above. In particular, Aquixtla is characterized by intensive greenhouse tomato production (Zamora-Islas *et al.* 2021), a productive framework relevant to interpreting the lower AChE activity recorded in this locality. This background allows us to consider exposure to anticholinesterase compounds as an additional hypothesis to explain the observed pattern.

Micronucleus frequency. The frequency of micronucleated cells was higher in Tlaxco (1.25%) than in Aquixtla (0.30%) and Nauzontla (0.23%), although the difference was statistically significant only between Tlaxco and Nauzontla. This pattern suggests greater cytogenetic damage in individuals from Tlaxco. The usefulness of the micronucleus assay in bats has been previously demonstrated. Benvindo-Souz *et al.* (2019) evaluated exfoliated cells of the buccal mucosa of *Nyctinomops laticaudatus*, *Noctilio albiventris*, and *Pteronotus parnellii*, and demonstrated that this tissue allows the detection of micronuclei and other nuclear alterations. Their findings support the use of this tissue as a biomarker of genetic damage in ecotoxicological studies of bats.

Our results are also consistent with those reported by Benvindo-Souz *et al.* (2019), who evaluated the frequency of micronuclei in buccal mucosal cells of various bat species and found a higher frequency of these alterations in insectivorous individuals from agricultural and urban areas than in those captured in conserved environments. Although the species and scenarios evaluated by these authors differ from those in the present study, both studies show that the frequency of micronucleated cells can vary among bat populations across different environmental conditions, supporting the validity of this parameter as a biomarker of genetic damage in ecotoxicological studies.

A similar pattern has been documented in studies that evaluated micronuclei in blood samples. Sandoval-Herrera *et al.* (2021) recorded a higher frequency of micronuclei in erythrocytes of *Pteronotus mexicanus* from roosts surrounded by a higher proportion of agriculture and anthropic environments. Subsequently, Sandoval-Herrera *et al.* (2023) found a consistent response of this biomarker in *Eptesicus fuscus* experimentally exposed to chlorpyrifos and in wild populations of *Pteronotus mexicanus* associated with varying levels of agricultural development. These studies align with our results, indicating that certain populations associated with agricultural landscapes may exhibit a stronger signal of genotoxic damage.

Unlike AChE activity, the higher frequency of micronucleated cells in Tlaxco is consistent with greater cytogenetic damage accumulated during the period of buccal epithelium renewal. The micronucleus assay integrates the effects of various agents with genotoxic potential, so the higher frequency observed in Tlaxco suggests greater exposure to this type of compounds compared to the other localities evaluated. However, this interpretation should also consider the physiological state of the individuals at the time of sampling. The Tlaxco individuals were captured in February, at the end of the hibernation period, while those from Nauzontla and Aquixtla were captured in May and July, respectively, during other stages of the annual cycle of *M. velifer*. The physiological differences associated with these stages may contribute to the variation observed in biomarkers and, therefore, constitute a relevant factor in interpreting the highest percentage of micronucleated cells recorded in Tlaxco.

The difference between Tlaxco and Nauzontla is particularly relevant because both localities exhibited similar proportions of agricultural cover, close to 47%, but differed in the presence of micronucleated cells. These findings indicate that the extent of agriculture alone does not explain the differences observed in this biomarker. A possible explanation is that differences in crops, active ingredients used, frequency of application, or temporal coincidence between phytosanitary management and foraging activity have led to greater genotoxic exposure in Tlaxco. In addition, the coexistence of agricultural activities and forest remediation actions could represent an additional source of exposure, especially if the latter included the use of pesticides to control bark beetles. Finally, it should be considered that the damage observed in the captured specimens could have originated prior to fieldwork in this study, at other locations used by bats during their seasonal movements.

In contrast, the lower percentage of micronucleated

cells recorded in Nauzontla could be consistent with a lower recent exposure to genotoxic agents, with the use of different products, with a lower coincidence between the applications and foraging activity of bats, or with a different use of agricultural areas. These possibilities show that a similar proportion of agricultural cover may correspond to different intensities, products, and routes of exposure.

Aquixtla showed intermediate values for micronucleated cells and did not differ significantly from Tlaxco or Nauzontla, suggesting an intermediate response that requires considering landscape features and individual variation together. Although this locality has a lower proportion of agricultural land and greater coverage of primary vegetation, intensive tomato production under greenhouses is characterized by frequent use of insecticides and fungicides, so the agricultural area does not necessarily reflect the intensity of phytosanitary management. The absence of statistical differences relative to the other locations may also be attributable to individual variability and sample size, so the interpretation of this pattern should consider both the productive characteristics and the physiological variation among individuals.

Finally, the time period represented by the buccal mucosa must be taken into account. Micronuclei originate from proliferative cells in the basal layer and are subsequently observed when they migrate to the surface during epithelial renewal (Thomas *et al.* 2009). Although the precise duration of this time window has not been determined for *M. velifer*, this consideration is relevant to our study because the individuals were captured in roots that are part of a system of seasonal movements between different locations.

Therefore, the micronucleated cells recorded in our study may reflect exposure events that occurred in the weeks prior to capture. Due to the seasonal movements of the species, some of the detected damage may have originated in other refuges or foraging areas previously used by individuals.

Land use and productive contexts. The analysis of land use allowed us to describe the general context of the landscape in each locality; Tlaxco and Nauzontla showed similar percentages of agricultural cover, close to 47%, while Aquixtla showed a lower proportion of agriculture and greater coverage of primary vegetation. Despite these differences in the landscape, Aquixtla and Nauzontla showed statistically similar AChE and micronucleated cell values. In contrast, Tlaxco had significantly higher AChE activity than both locations and a significantly higher percentage of micronucleated cells than Nauzontla. This pattern shows that the extent of agricultural land should be interpreted as a general descriptor of the landscape and complemented by information on pesticide use intensity and bat exposure routes.

Although the mapping allowed for the characterization of the spatial distribution of agricultural land, it does not provide information on the active ingredients used, the frequency of application, or the intensity of phytosanitary management. Therefore, localities with similar agricultural land cover may represent different potential exposure scenarios. In Tlaxco, agriculture coexists with forestry activities and sanitation programs aimed at controlling bark beetles (Comisión Nacional Forestal 2023), which could have constituted an additional source of pesticide exposure during the study period. Therefore, these activities should also be considered when interpreting potential sources of exposure in the locality. In Aquixtla, intensive greenhouse tomato cultivation is an important component of the local production system (Zamora-Islas *et al.* 2021), whereas agricultural systems that include corn, potatoes, and coffee predominate in Nauzontla (Ayuntamiento del Municipio de Nauzontla 2025). In addition, consultations with producers, workers, and distributors of agro-inputs documented the use of chlorpyrifos, methyl parathion, carbofuran, carbaryl, and cypermethrin in the study localities. Although this information does not allow estimating doses, application frequencies, or exposure levels, it provides a consistent context for interpreting observed differences in biomarkers and guiding future chemical and environmental analyses.

In insectivorous bats, ingestion of contaminated prey is one of the main potential routes of pesticide exposure. The coincidence between applications, insect availability, and nocturnal foraging activity can favor the indirect intake of these compounds, even when bats are not actually foraging in crop areas during pesticide applications (Stahlschmidt *et al.* 2017). In this context, differences in production systems, prey origins, use of feeding areas, and movements around roosts provide an ecological framework that helps explain why localities with similar agricultural proportions exhibited different responses in the biomarkers evaluated.

Finally, the interpretation of the biomarkers must also consider the temporal dimension of the sampling and the seasonal function of the roosts used by *Myotis velifer*. The individuals were captured at different times in their annual cycle and in roosts that serve different biological functions, implying possible differences in their physiological state and recent exposure history (Villa-Ramírez 1966; Arratia 2000). In addition, due to seasonal movements of the species, observed responses may reflect exposures that occurred at other roosts or at foraging areas used prior to capture. Together, these factors highlight that biomarker responses reflect the interaction among the environmental conditions of each locality, the recent history of exposure, and the physiological state of individuals.

Conclusions

Bats from Tlaxco showed the highest values for both biomarkers, although each reflected a different biological process. While the higher percentage of micronucleated cells indicates more pronounced relative cytogenetic damage compared to Nauzontla, the high AChE activity in Tlaxco represents a differential enzymatic response between localities that cannot be directly interpreted as

evidence of pesticide exposure. Overall, the differences observed among localities must be interpreted considering the time of the sampling, the physiological state of individuals, and the environmental characteristics of each locality, in addition to the land-use context.

The comparison between Tlaxco and Nauzontla also showed that localities with similar agricultural proportions may present different biological responses. This highlights the importance of considering, in addition to agricultural extension, the characteristics of productive management, potential routes of exposure, and the species' ecology. The joint evaluation of AChE and micronuclei allowed for the characterization of distinct biological responses among the studied localities and provided a comparative framework for the variation of both biomarkers in *Myotis velifer*, as well as the observed values of plasma AChE activity and the frequency of micronucleated cells in individuals from these three locations.

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Declaration of Artificial Intelligence use

The authors declare that ChatGPT was used to obtain suggestions regarding the style and clarity of the manuscript, as well as for grammatical and syntactic revision.

Author contributions

Angélica M. Orozco-Robles: Conceptualization, investigation, methodology, formal analysis, visualization, writing—original draft, review, and editing. Miguel A. León-Galván: Conceptualization, methodology, supervision, project administration, resources, review, and editing. Noé Salinas-Arreortua: Conceptualization, methodology, supervision, resources, review, and editing. Gihovani A. Sámano-Barbosa: Investigation, methodology, review, and editing. Luis M. Guevara-Chumacero: Conceptualization, methodology, supervision, resources, review, and editing.

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