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Micronucleus Test Reveals Genotoxic Effects in Bats Associated with Agricultural Activity

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Abstract: Bats play a vital role in our ecosystems and economies as natural pest-control agents, seed dispersers, and pollinators. Agricultural intensification, however, can impact bats foraging near crops, affecting the ecosystem services they provide. Exposure to pesticides, for example, may induce chromosome breakage or missegregation that can result in micronucleus formation. Detection of micronuclei is a simple, inexpensive, and relatively minimally invasive technique commonly used to evaluate chemical genotoxicity but rarely applied to assess wildlife genotoxic effects. We evaluated the suitability of the micronucleus test as a biomarker of genotoxicity for biomonitoring field studies in bats. We collected blood samples from insectivorous bats roosting in caves surrounded by different levels of disturbance (agriculture, human settlements) in Colima and Jalisco, west central Mexico. Then, we examined the frequency of micronucleus inclusions in erythrocytes using differentially stained blood smears. Bats from caves surrounded by proportionately more (53%) land used for agriculture and irrigated year-round had higher micronucleus frequency than bats from a less disturbed site (15% agriculture). We conclude that the micronucleus test is a sensitive method to evaluate genotoxic effects in free-ranging bats and could provide a useful biomarker for evaluating risk of exposure in wild populations. *Environ Toxicol Chem* 2021;40:202–207.

Keywords: Wildlife ecotoxicology; Biomarkers; Insectivorous bats; Biomonitoring; Risk assessment

INTRODUCTION

Intensification in land use for farming and the proximity of crop fields to natural environments have increased the risk of wild species coming into contact with sprayed pesticides and potentially suffering from their toxic effects (Berny 2007; Williams-Guillén et al. 2015). Commonly used agrochemicals such as organic pesticides have been shown to be genotoxic agents, inducing damage to genetic material such as gene mutation, chromosomal alterations, and DNA damage (Bolognesi 2003). These genetic alterations can cause cell death or induce malignancies that affect the organism's function and could eventually reduce survival (Sailaja et al. 2006; Phillips and Arlt 2009). Unlike most molecular changes, chromosome breakage and missegregation represent irreversible genetic damage, which can be readily detected in exposed organisms using simple techniques (Amiard and Amiard-Triquet 2013).

The micronucleus test, for example, is used to detect damage at the chromosomal level and offers an inexpensive and minimally invasive technique to evaluate genotoxicity in animals (Araldi et al. 2015). The test is based on the detection of small, rounded inclusions, called "micronuclei," that are readily visible under light microscopy (Samanta and Dey 2012). These are chromosome fragments or whole chromosomes that are not incorporated into the principal nucleus of a daughter cell during nuclear division (Bonassi et al. 2007). With new technology, more sophisticated methods to evaluate genotoxicity have become popular such as the comet assay (Olive and Banáth 2006) and the mouse lymphoma assay (Clements 2000). These methods, however, require expensive techniques like flow cytometry, gel electrophoresis, and cell cultures that might not be widely available or accessible, especially under field conditions. On the other hand, the traditional micronucleus assay requires simple technology like light microscopy and allows for simultaneous evaluation of additional biomarkers, such as differential white blood cell count and red blood cell profiles, using the same blood smear sample (Davis and Maney 2018). Thus, the assay is field-compatible, reduces

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animal handling, and allows multiparameter data acquisition. The formation of micronucleus has been extensively used as a biomarker of genotoxicity in humans, model organisms, and recently wildlife (Shepherd and Somers 2012; Baesse et al. 2015; Souto et al. 2018; Benvindo-Souz et al. 2019; Benvindo-Souza et al. 2019). However, only a few studies have investigated mammalian responses to pollutant levels occurring in the environment, and many focus on the acute poisoning events rather than the chronic daily exposure of animals inhabiting agricultural lands (Köhler and Triebskorn 2013).

Insectivorous bats, for example, are susceptible to pesticide exposure and its deleterious effects because they forage in agricultural lands and prey on potentially contaminated pests (Stahlschmidt et al. 2017). Direct exposure to pesticides in bats has not been quantified but could be significant because pesticide application often takes place at dusk when bat mobility and activity increase (Bayat et al. 2014). Exposure to agricultural contaminants may have long-term sub-lethal consequences that can negatively impact the populations of bat species foraging in croplands (Bayat et al. 2014). Even at low doses, pesticide exposure can cause neurological and physiological effects in nontarget vertebrates, such as immunosuppression, endocrine disruption, reproductive failure, and altered behavior (Bayat et al. 2014). Despite their importance in ecosystems as pest-control agents, seed dispersers, and pollinators, bat populations have decreased worldwide; and this is likely due to anthropogenic activities (Kunz et al. 2011). Various studies have advocated for bats as potential bioindicators because of their wide geographical range, sensitivity, and diverse ecology (Jones et al. 2009). Their ecological importance and risk of exposure to genotoxic substances make bats a relevant group to study.

We sought to evaluate the suitability and efficacy of the micronucleus test in blood cells as a biomarker of genotoxic damage in wild bats that are potentially exposed to variable amounts of pesticides from croplands. We used the micronucleus test standardized in model organisms and humans to analyze blood smears collected in the field. We predicted that the differential agricultural management (e.g., seasonal vs year-round cultivation) and consequent potential exposure to genotoxic pesticides will be reflected in the number of micronuclei in the erythrocytes of wild bats. In addition, we hypothesized that individual sex might explain additional intraspecific variability. We predicted that insectivorous bats roosting in caves surrounded by a high proportion of farmed area year-round would present a higher frequency of micronuclei compared to bats roosting in caves with a relatively lower proportion of surrounding area farmed and/or less intensively farmed. Based on several studies suggesting that male mice are more susceptible to genotoxic effects than females (Hamada et al. 2003; Heuser et al. 2008; Rojas-Lemus et al. 2014), we also predicted that female bats would present a lower frequency of micronuclei than males because of the genotoxic protective mechanisms associated with female reproductive hormones (Nagae et al. 1991).

MATERIALS AND METHODS

Study site

We captured a total of 35 bats from 3 different cave-roosting colonies in Colima and Jalisco, west central Mexico (Figure 1). The predominant vegetation in this area is tropical deciduous and semideciduous forests. All bats were collected in March 2019 (dry season) using hand-nets and mist-nets set in each cave site. We sampled bats from 3 caves surrounded by different levels of agricultural intensity: Don Pancho, El Salitre, and La Fábrica Caves (Figure 1). Don Pancho Cave is located on San Agustin island, 1 km off the coast on Chamela Bay, Jalisco (19.535°N, −105.088°W). El Salitre Cave is 3.6 km south of Los Ortices village, Colima (19.083°N, 103.726°E). La Fábrica Cave is 6.4 km southwest of Coquimatlán town, Colima (19.151°N, −103.835°W). All sites had croplands in their proximity because agriculture is practiced extensively throughout this region, mainly fruit crops (e.g., lemon and papaya) in Colima and coconut palm in the coast of Jalisco (Servicio de Información Agroalimentaria y Pesquera 2018). Using spatial analysis in QGIS software (QGIS Development Team 2020), we calculated the proportion of land dedicated to agriculture within 2 km around each cave. This buffer area covers the average home range for many neotropical bat species and has been used elsewhere (Meyer and Kalko 2008; Ferreira et al. 2017). For the analyses, we used the most recent land-use coverage layer publicly available by the Sistema Nacional de Información Estadística y Geográfica of Mexico, which is based on Landsat data (Instituto Nacional de Estadística Geografía e Informática 2017). We used the agriculture land extension within the buffer as a proxy of the potential pesticide exposure that the surrounded area could represent for the bats roosting close by. Because genotoxic agents might also come from human settlements, we also used the global Human Modification Index (HMI; Kennedy et al. 2019) as a measure of the general level of disturbance. The HMI is a cumulative measurement with possible values between 0 (no disturbance) and 1 (highest disturbance) that includes transportation, human settlement, agriculture, extractive activities, and electric infrastructure (Kennedy et al. 2019). We used both agricultural extension and the HMI to categorize pesticide exposure of the sites as low, intermediate, and high. El Salitre Cave was classified as a low-exposure site with 17% crop coverage and HMI = 0.40, Don Pancho Cave was categorized as an intermediate-exposure site with 28% crop coverage and HMI = 0.47, and La Fábrica Cave was classified as a high-exposure site with 53% crop coverage and HMI = 0.54.

Study species

Pteronotus mexicanus (family Mormoopidae) is a medium-sized neotropical bat with body mass of 10 to 20 g and forearm length of 5.3 to 5.7 cm (Herd 1983). It is distributed throughout Mexico, Central America, the Antilles, the Guyana Shield, and the Amazon (Herd 1983; Clare et al. 2013). This species is

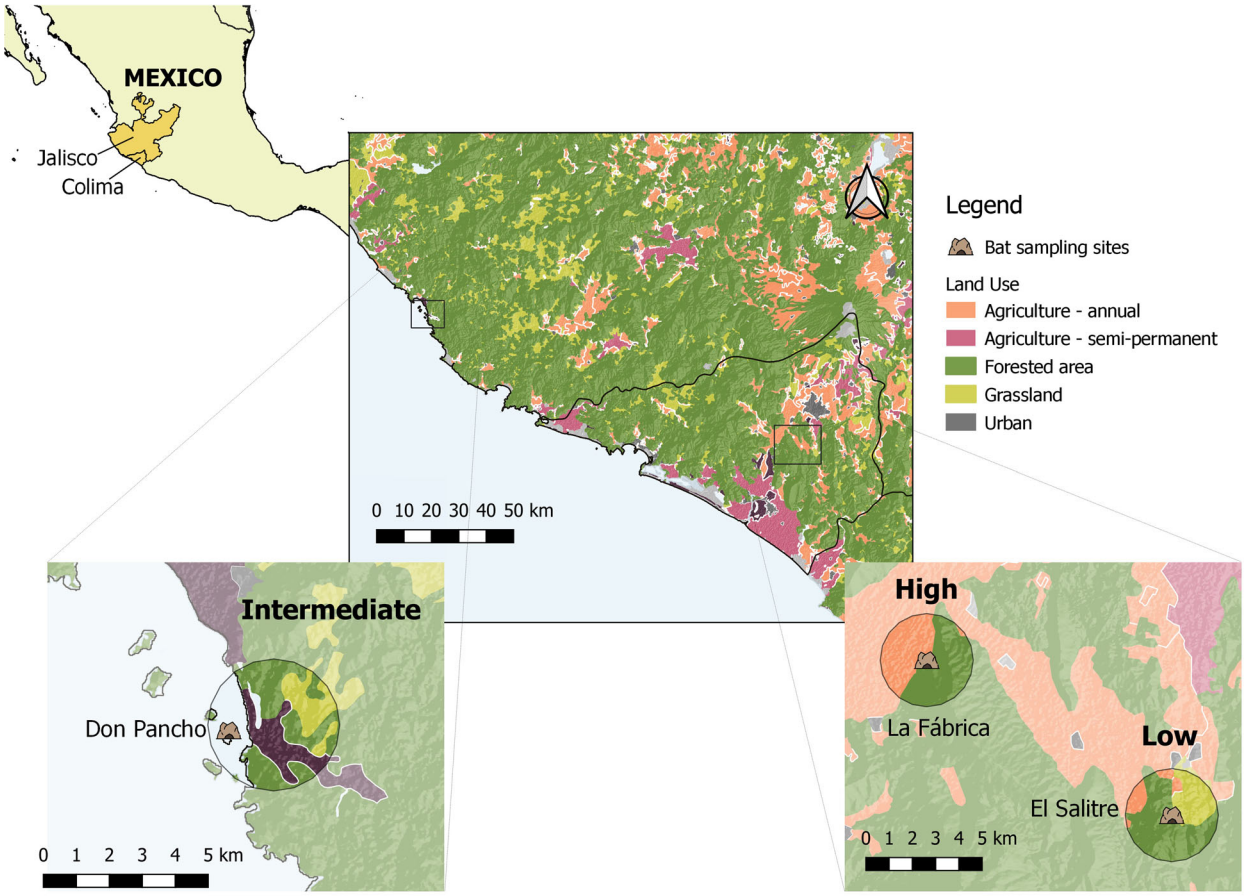

FIGURE 1: Depiction of the study site in Colima and Jalisco, west central Mexico, showing the 3 cave sites where the bats were captured. Source: Instituto Nacional de Estadística Geografía e Informática (2017).

commonly called Parnell's mustached bat, and individuals are regularly found in a variety of forest types ranging from lowland rainforest to drier forest and open landscapes such as crop fields (De Oliveira et al. 2015). Whereas most bats prefer to forage along river channels because of reduced structural hindrance and interference with bat flight patterns (Ober and Hayes 2008), *P. mexicanus* is more active in areas with dense vegetation and feeds mainly on flying insects: Hemiptera, Diptera, Coleoptera, and Hymenoptera (De Oliveira et al. 2015). We selected Parnell's mustached bat as a target species for 1) its relatively large size that facilitates blood collection; 2) its recorded foraging in croplands; 3) its habit of consistently using the same roost, allowing us to assume that it forages consistently in this locality; and 4) its wide distribution in the neotropics (Herd 1983; Clare et al. 2013; De Oliveira et al. 2015).

Animal ethics statement

Field procedures followed the guidelines for safe and humane handling of bats published by the American Society of Mammalogists (Sikes 2016) and were approved by the Institutional Animal Care and Use Committee of the University of Toronto (20012113). Sample collection was approved under permit FAUT-0069.

Micronucleus assay

We drew approximately 2 μL of blood from the radial artery of each bat and smeared the aliquot onto a clean glass microscope slide. The slides were air-dried, fixed with methanol, and stained using a Hem 3™ Rapid staining kit (Fisher HealthCare; 122911). A code was applied by a different experimenter to each slide such that the person who analyzed the blood smears was blinded to site information. All smears were read by the same person. The criteria for micronucleus identification were based on Schmid (1976): small, basophilic, round inclusions inside the erythrocytes (Figure 2). Because we conducted a differential counting of leukocytes simultaneously, we counted the number of micronuclei using the method of "battlement track," covering the entire width of the blood smear until reaching 100 leukocytes (Houwen 2001). We assumed a 1:2000 leukocyte to erythrocyte ratio to estimate the number of micronuclei per 1000 erythrocytes (Liudmila et al. 2017). The relative micronucleus count was scored in peripheral erythrocytes, which included both normochromatic

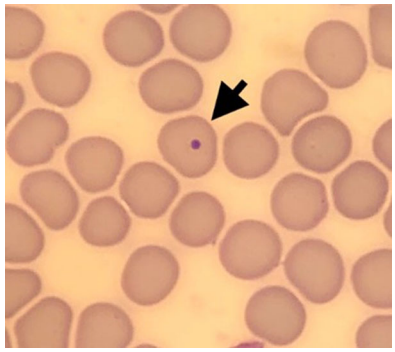

FIGURE 2: Magnified (×1000) detail of blood smear from *Pteronotus mexicanus* presenting a micronucleated erythrocyte indicated by the arrow.

(mature) and polychromatic (immature) erythrocytes (Naidoo et al. 2015). Like cell counts, photo documentation was conducted using a light microscope under oil immersion at a magnification of ×100. The assay was performed in accordance with the US Environmental Protection Agency (1998) guidelines specific for mammals.

Data analysis

We performed square-root transformation on our data such that a Poisson distribution resulted ($\chi^2 = 8.95$, $p = 0.9608$). To analyze the effect of site, sex, and the interaction on the frequency of micronuclei, we used a generalized linear model with a Poisson distribution. Next, we conducted a pairwise comparison using Bonferroni correction. All analyses were performed with R studio (Ver 3.6.2), using a level of significance of $p < 0.05$ (R Development Core Team 2019). Values are presented as the median ± quartile deviation.

RESULTS

We screened a total of 35 blood smears of *P. mexicanus*. One individual did not present any micronuclei in the analyzed peripheral blood sample. The median proportion of erythrocytes containing micronuclei was 0.065 ± 0.048, with a range between 0 and 0.355. The median proportions of cells containing micronuclei were 0.010 ± 0.005 in samples from the low-exposure site, El Salitre ($n = 11$); 0.070 ± 0.044 in samples from the intermediate-exposure site, Don Pancho ($n = 11$), and 0.100 ± 0.065 in samples from the high-exposure site, La Fábrica ($n = 13$). Controlling for all other variables, we found that roosting site ($\chi^2 = 28.252$, $df = 2$, $p < 0.001$) was an informative predictor of micronucleus frequency. Bonferroni correction revealed that bats roosting in El Salitre (low-exposure site) presented a significantly lower frequency of micronucleated peripheral erythrocytes in comparison with bats captured from Don Pancho ($p = 0.037$; mid-exposure site) and La Fábrica ($p < 0.001$; high-exposure site; Figure 3). However, we found no evidence that sex ($\chi^2 = 0.314$, $df = 1$, $p = 0.577$) or the interaction term ($\chi^2 = 0.021$, $df = 2$, $p = 0.991$) was an informative predictor of micronucleus frequency.

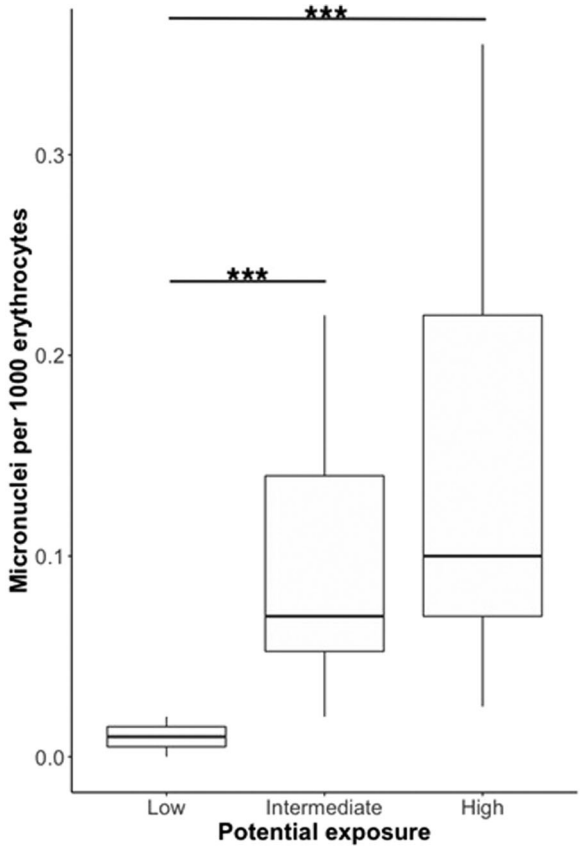

FIGURE 3: Frequency of micronuclei of *Pteronotus mexicanus* captured from El Salitre (low), Don Pancho (intermediate), and La Fábrica (high). The median is indicated by the horizontal line in the middle of each box and the interquartile range by the vertical lines. ***Significant differences ($p < 0.05$, Bonferroni correction) between samples.

DISCUSSION

In the present study, we provide support for the suitability of micronucleus frequency as a biomarker of toxicity in free-ranging bats. As we predicted, we found that bats roosting in sites surrounded by higher proportions of agricultural lands, with likely greater exposure to pesticides, present higher frequency of micronuclei compared with bats inhabiting sites with lower agricultural activity (Figure 3). The sensitivity of micronucleus frequency as a biomarker of genotoxicity is supported by the highly significant results, even with a relatively small sample size. We hypothesize that elevated micronucleus is due to increased pesticide exposure; however, testing this hypothesis would require an accurate quantification of pesticides in the environment at each of the sampling sites and considering different routes of exposure (i.e., air, food, water).

Research on bat ecotoxicology is limited, and the use of genotoxic biomarkers is especially rare (Oliveira et al. 2020). Studies looking at chromosomal aberrations and DNA damage using the comet assay and flow cytometry did not find a correlation between environmental pollutants and genotoxic effects in wild bats (Thies et al. 1996; Naidoo et al. 2015). Nevertheless, the few studies that have evaluated micronucleus frequency as an endpoint have found similar results to those

reported in the present study, where high frequencies of micronuclei were observed in populations inhabiting human-altered environments (Benvindo-Souz et al. 2019; Benvindo-Souza et al. 2019). Notably, one of these studies looking at frugivorous and insectivorous species found that the genotoxic risk might be influence by the bats' diet and the type of disturbance (urban vs croplands) they are exposed to (Benvindo-Souza et al. 2019), suggesting that ecological factors like dietary guild should be considered in future studies and selection of bioindicator species.

Part of the intraspecific variability observed could be explained by sex differences in genotoxic sensitivity. Even though previous studies on bats did not find an influence of sex on the frequency of micronuclei (Benvindo-Souz et al. 2019), these studies looked at oral epithelium rather than erythrocytes. As has been reported in mice, we expected that female bats would present lower micronucleus incidence than males because of the protective effect of estrogen on erythroblasts and reduction in erythropoiesis (Nagae et al. 1991). However, our data showed no significant difference in micronucleus frequency between sexes.

The micronucleus test is a widely used method in laboratory-based studies for assessing chromosomal damage because it reliably measures both chromosome loss and chromosome breakage (Fenech 2000). Because micronucleus frequency is an index of accumulated genetic damage during the life span of the cells, the tissue being examined will constrain the time window of genotoxic exposure that is being evaluated. Recent studies on bats have successfully implemented the micronucleus test in exfoliated cells of the buccal mucosa (Benvindo-Souz et al. 2019; Benvindo-Souza et al. 2019). This test in buccal mucosa provides a snapshot of genotoxicity exposure occurring 1 to 3 wk prior to data collection (Stich et al. 1983; Thomas et al. 2009), whereas blood cells convey information about an approximately 3-mo retrospective period (Voigt et al. 2003). Although collecting buccal mucosa is less invasive for the animal (Torres-Bugarín et al. 2014), using standard blood smears allows for evaluation of other physiological parameters relevant for health assessment of bats such as leukocyte profiles and endoparasites (Davis and Maney 2018). Hence, depending on the goal of the study and the access to samples in the field, researchers should consider the turnover rate of different tissues when selecting a method that fits their needs better. For example, when monitoring pesticide exposure and samples can be accessed immediately, the micronucleus test on buccal mucosa could provide superior detection. Conversely, if researchers aim to assess general levels of genotoxic stress, as well as employ additional blood analyses, at seasonal (~3-mo) intervals; then, blood sampling and subsequent application of the micronucleus test would likely provide more comprehensive information.

More accurate and sophisticated methods have been developed to detect and identify genotoxic exposure and effects in animals such as the comet assay (Olive and Banáth 2006) and mouse lymphoma assay (Lloyd and Kidd 2012). These techniques (e.g., flow cytometry) are not necessarily more sensitive at detecting effects of agricultural activities but do require expensive equipment and training. Access to these resources might be limited for institutions with less financial support such as conservation nongovernmental organizations and researchers in developing countries. The use of early warning biomarkers such as genotoxic effects is most needed in these regions, where the use of pesticides is extensive and there is a high biodiversity. Particularly for these and other field-based researchers, we proposed use of the micronucleus test as a logistically simple, sensitive, and robust method for biomonitoring genotoxic effects in wild bat populations.

Supplemental Data—The Supplemental Data are available on the Wiley Online Library at https://doi.org/10.1002/etc.4907.

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Author Contributions—N. Sandoval-Herrera: study conception, sample collection; J.P. Castillo: sample analysis; N. Sandoval-Herrera and J.P. Castillo: data analysis, writing; K.C. Welch and L.G.H. Montalvo: secured funding for field and laboratory work. All authors reviewed drafts of the manuscript.

Data Availability Statement—Data, associated metadata, and calculation tools are available from the corresponding author (natalia.sandovalherrera@mail.utoronto.ca).

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