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Research Article

A method to quantify genomic damage in mammal populations

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Abstract

The Buccal Micronucleus Cytome (BMCyt) assay is a relatively non-invasive method successfully applied to evaluate genomic damage for biological monitoring of human populations exposed to various mutagenic and carcinogenic compounds. This paper presents a protocol developed to apply the buccal micronucleus assay to wild mammal species during field or management activities. We applied the BMCyt to one population of Eastern grey squirrels (*Sciurus carolinensis*, n=37) and one of wild boars (*Sus scrofa*, n=41) living in anthropized and natural areas, respectively. Micronuclei, nuclear buds, and other nuclear abnormalities, e.g. binucleated, Kidney-shaped nucleus, broken eggs, karyorrhexis, cells with condensed chromatin, pyknotic and karyolysis, were recorded. The frequencies of micronuclei and nuclear buds were respectively 0.51±0.60 and 1.29±1.08 for wild boars and 0.16±0.37 and 0.57±0.73 for grey squirrels. Sex, age classes and body weight had generally no effects on the frequencies of genomic damages, except for a limited effect of weight on broken eggs and karyolitic cells in grey squirrels and of sex on condensed chromatin in wild boars. In conclusion, the results of this study indicate that buccal mucosa is a sensitive site for detecting micronuclei and other nuclear abnormalities and that the protocol used in this paper may represent a valuable tool to evaluate the genomic damage in wild species living in both anthropized and natural areas.

Introduction

Human pressure on the environment continuously increases (Venter et al., 2016), threatening species and ecosystems (Dirzo et al., 2014; Johnson et al., 2017). In Europe, primary drivers of landscape changes are urbanization, infrastructure development and agricultural intensification, which reduce natural habitats and their diversification.

While many species are in decline, especially in lowlands where human pressure is higher (Donald et al., 2006), others have adapted to human-altered habitats enlarging their range and getting closer to urban areas or surviving in intensive agro-ecosystems (Wesolowski and Fuller, 2012; Ciach and Fröhlich, 2019). These species have higher ecological plasticity and resilience to human disturbance; however, occupying human-altered environments, they are increasingly exposed to several stressors associated with an increasing human footprint.

It is very likely that environmental xenobiotics, which affect human health, have similar effects on animal populations living in the same context. One known effect of environmental pollutants in humans is the increase in their genomic instability affecting the baseline values of cytogenetic markers (Santovito et al., 2018, 2020b). Genomic instability results from damage to DNA that occurs during cell division and is not repaired (López-Otín et al., 2013). Many studies have shown a correlation between an increased rate of genomic damage and diseases such as cancer in humans (Kasabwala et al., 2022; Guscott et al., 2022; Tammewar and Gadkari, 2022). Therefore, evaluating control baseline data for cytogenetic markers in human and animal populations may be crucial for the general health risk assessment and as a general guideline to estimate the potential risk in other wild populations exposed to similar pollutants.

The Buccal Micronucleus Cytome (BMCyt) assay is a method developed for studying DNA damage, chromosomal instability, cell death and the regenerative potential of human buccal mucosal tissue (Thomas et al., 2009). Micronuclei (MNi) represent genomic damage to oral epithelium's basal layer cells, which originate from acentric chromosome fragments or whole chromosomes that fail to segregate correctly during mitotic division and appear in the cytoplasm of interphase cells as small additional nuclei (Kuprina et al., 2021). Therefore, they represent clastogenic damage due to chromosome/chromatid breaks induced by xenobiotics and aneugenic damage as a consequence of agents that interfere with the mitotic apparatus leading to missegregation of whole chromatids or chromosomes during mitosis. In both cases, the chromatin is not properly distributed to the daughter nuclei and remains in the cytoplasm as a micronucleus (Fenech, 2007; Kirsch-Volders et al., 2011; Hintzsche et al., 2017). For these reasons, micronuclei are used as biomarkers of the genomic damage caused by exposure to genotoxic agents in vertebrates, humans included (Thomas et al., 2009; Benvindo-Souz et al., 2019a,b; Shepherd and Somers, 2012). The baseline for micronucleated buccal cells in healthy human subjects not exposed to genotoxic agents is usually withi0n the range of 0.30-1.70 per 1000 cells (Bonassi et al., 2011). It is known that many environmental xenobiotics can increase this baseline frequency and, consequently, the risk of cancer diseases and the incidence of other pathologies (Fenech et al., 2021).

Chromosomal instability may be measured by evaluating also nuclear buds (NBUDs), representing the elimination process of amplified DNA and excess chromosomes from aneuploid cells (Bolognesi et al., 2013). Moreover, the oral epithelium's basal cell layer (stratum basale) contains stem cells that are continuously dividing, and some of them may degenerate into cells with condensed chromatin, fragmented nuclei (karyorrhectic cells), pycnotic nuclei, or may completely lose their nuclear material (karyolitic or "ghost" cells). In some cases, cells may

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be blocked in a binucleated stage or exhibit broken eggs, representing biomarkers of gene amplification (Holland et al., 2008). Criteria for identifying and scoring cell types with MNi, NBUDs and other nuclear rearrangements are reviewed in Bolognesi et al. (2013) and Thomas and Fenech (2011).

The Micronucleus test in exfoliated cells of the buccal mucosa has been successfully used to evaluate the impacts of environmental stressors on humans, such as atmospheric pollution (Panico et al., 2020; Acito et al., 2022), dust particles (Wultsch et al., 2019), pesticides (Cobanoglu et al., 2019; Dos Santos et al., 2022) and heavy metals (Santos et al., 2023; Nagaraju et al., 2022). The same protocol already consolidated in humans is replicable for different taxa, such as fish (Walton et al., 1988), birds (Shepherd and Somers, 2012), wild mammals (Benvindo-Souz et al., 2019a,b; Benvindo-Souza et al., 2022), as well as those mammals used in laboratory experiments such as rodents (Thomas et al., 2009; Çavuşoğlu et al., 2009). Recently, the MNi assay has also been successfully applied in domestic dogs and cats (Santovito et al., 2022), and in invertebrates (Santovito et al., 2020a; Piccini et al., 2023).

In this scenario, our laboratory is developing protocols to apply the Buccal Micronucleus Cytome (BMCyt) assay to different wild populations, e.g. mammals, amphibians, and invertebrates (Santovito et al., 2020a). Control activities of native or alien species and some field research offer the possibility to manipulate many animals and could represent an opportunity to collect samples for studies on DNA damage in wild mammals. We present here a protocol developed to apply BMCyt to wild mammals during field research or management activities. We took advantage of a wild boar (*Sus scrofa*) control plan and research on the Eastern grey squirrel (*Sciurus carolinensis*) introduced to Italy (Bertolino et al., 2014).

Materials and Methods

Study species

We applied BMCyt assay to one population of Eastern grey squirrel and one of wild boar. The Eastern grey squirrel is an invasive alien species introduced in many parts of Italy (Loy et al., 2019). The Eastern grey squirrel population in Piedmont is the largest in Italy and originated from the first introduction in the country (Bertolino et al., 2014; Loy et al., 2019). The Eastern grey squirrel is listed in the Invasive Alien Species of Union concern list connected to the European Regulation 1143/2014. The species included on this list are subject to restrictions on keeping, importing, and breeding and the Member States are required to take measures for eradication or control. We sampled a population living in Moncalieri, a suburban area of the city of Turin (Piedmont region, Italy), taking advantage of an ongoing study which provides for the euthanasia of animals. Wild boars were sampled in La Mandria Natural Park, a regional protected area part of the "Aree Protette dei Parchi Reali". The park management body is carrying out a control plan on this overabundant species, and park rangers have agreed to collect samples immediately after shooting the animals.

Field protocol

Exfoliated buccal mucosa cells were collected in dead animals by scraping, for at least 1 min, the mucosa of the inner lining of one or both cheeks with a toothbrush with varying tip sizes depending on the mouth dimension of sampled animal. We used the smallest toothbrush (for child aged 0-3 years) with squirrels (Fig. 1A), and for wild boar a standard adult toothbrush (Fig. 1C). Buccal cells were also collected from the inner side of the lower lip and palate. Indeed, the variability in MNi frequency between these two buccal areas was found to be minimal for control subjects (Holland et al., 2008).

The tip of the toothbrush was immersed in a 50 mL tube containing 15-20 mL of a fixative solution consisting of methanol and acetic acid 3:1 (Fig. 1B). After shaking for at least 1 minute to detach as many cells as possible from the bristles, the tube with the fixative solution containing the cells was stored at room temperature prior to analysis. Other published protocols involve a passage in a physiological solution before fixing the cells, in order to detach the cells, and then a second passage in fixative. In our experience, we observed no substantial differences between the protocols and chose to skip the passage in the physiological solution. Another advantage of putting cells directly in contact with the fixative solution is that the cells are immediately fixed, avoiding any problems related to lysis and storage conditions.

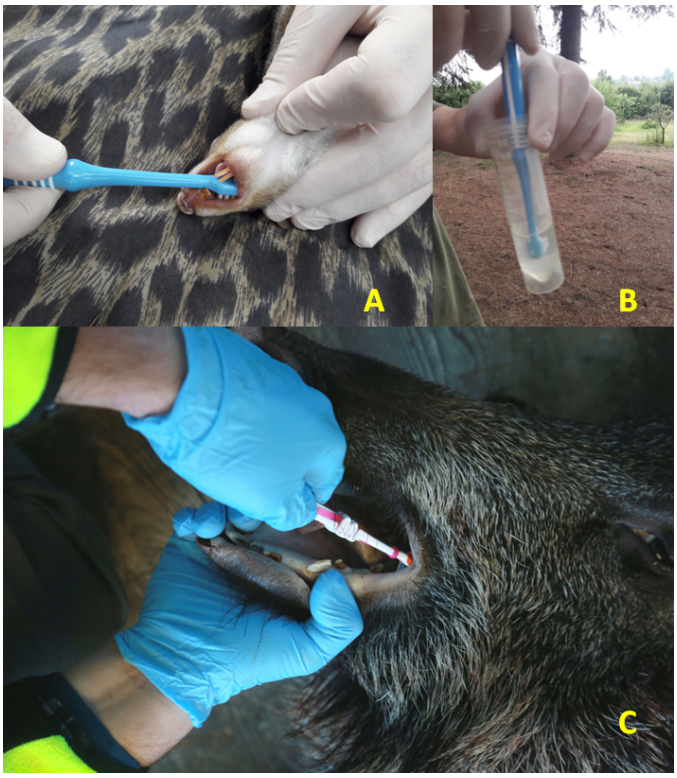

Figure 1 – (A) Buccal cell sampling with a small toothbrush (for kids age 0-3 years) on a grey squirrel; (B) immersion of the toothbrush in the fixative solution for cell release; (C) buccal cell sampling with an adult toothbrush on wild boar.

Laboratory analyses

In the laboratory, cells were collected by centrifugation, the supernatant was discarded, and the pellet was dissolved in a minimal amount of fixative, which was seeded on the slides to detect MNi, NBUDs, and other nuclear rearrangements by conventional staining with 5 % Giemsa (pH 6.8) prepared in Sörensen buffer (50 % of 67 mM Na_2HPO_4 + 50 % of 67 mM KH_2PO_4). Giemsa staining protocol has the advantage that it is fast and does not require a fluorescence microscope. However, Giemsa also positively stains keratohyalin granules or bacteria, which may give rise to an unwanted increase in false positives (Bolognesi et al., 2013) and, thus, requires experience in observing MNi under the microscope. In general, visual scoring is the most critical methodological step in the BMCyt assay, based on the subjective evaluation of the cytological parameters, which requires great experience by the scorer. For example, bacteria are commonly found in the mouth, especially in wild animals, but they can be differentiated from MNi by their characteristic shape, smaller size, color, and different staining intensity. Other structures that may represent confounding factors are small dye granules that may resemble MNi but usually have a slightly different refractility and color intensity (Holland et al., 2008).

Microscopic analysis was performed at 1000X magnification on a Leica Dialux 20 light microscope. MNi, NBUDs and other nuclear rearrangements were scored in 1,000 cells with well-preserved cytoplasm per subject according to the standard criteria for MNi evaluation (Bolognesi et al., 2013).

In order to be included into the MNi frequency count, cells should have the following characteristics: (a) intact cytoplasm; (b) little or no overlap with adjacent cells; (c) little or no debris; and (d) nucleus normal and intact with nuclear perimeter smooth and distinct (Bolognesi et al., 2013; Holland et al., 2008). The suggested criteria for identi-

fying MNi are: (a) rounded smooth perimeter and with a membrane; (b) similar structure and no more than 1/16 – 1/3rd of the diameter of the associated nucleus, (c) they must not be connected with the principal nucleus, (d) they must show the same intensity of staining and focal plane as nucleus and (d) absence of overlap with, or bridge to, the nucleus (Fenech, 2007).

We recorded all abnormalities, including MNi, NBUDs, nuclei with indentation (also called kidney-shaped nuclei due to their bilobed shape which resembles that of a kidney), broken eggs, condensed chromatin, pycnotic nucleus, binucleated cells, and karyolitic cells. Total damage represents the sum of these abnormalities, except for binucleated and karyolitic cells.

Statistical analysis

The counts of abnormalities are presented as the mean frequency (±standard error, SE) in a sample of 1000 cells. Differences in the body weight of sampled animals according to sex or age classes were evaluated with t-test or non-parametric Mann-Whitney tests according to the data distribution. We used two age classes: adults and juveniles/subadults (hereinafter juveniles). Squirrels were classified as juveniles and adults according to a combination of body weight and reproductive conditions (Santicchia et al., 2018); for wild boar, we used the dental eruption tables as a reference (Monaco et al., 2010), taking as cut-off an age of 15 months.

We used Generalized Linear Models (GLMs) to evaluate if age, sex and body weight of animals influenced abnormalities frequencies. Since the response variables were the number of each abnormality in 1,000 cells, we used GLMs with a Poisson distribution. We assessed the ratio variance/mean, the residuals of the full model for influential points and outliers, and the overdispersion parameter as the scaled Pearson's χ^2 estimator. In case of over or underdispersion, data were modelled with a negative binomial distribution with a log link. Since zero were common in the database, we also considered the possibility of zero-inflated models. In these cases, we compared poisson, negative binomial, zero-inflated models using an AIC approach, selecting the model with the lowest value (Supplemental material Table S1). Sex and age classes were considered factors, and body weight as a covariate. Model selection was based on the Akaike information criterion (AIC) using a stepwise backward procedure (Zuur et al., 2007). All the analyses were performed using R software (R Core Team, 2015) with the packages 'MASS' (Venables and Ripley, 2002) and 'pscl' (Jackman, 2020).

Results

We collected 37 squirrel and 70 wild boar samples. Observing the slides under a microscope, we realised that 29 boar samples were not readable due to the lysis of the cells. Therefore, we retained 41 (58.6 % of the total) wild boars and all (100 %) squirrel samples for further analysis.

We observed different aberrations, including micronuclei, nuclear buds, Kidney-shaped nucleus, broken eggs, condensed chromatin, pycnotic nucleus, binucleated and karyolitic cells. Examples of observed nuclear abnormalities are shown in Figure 2. Frequencies of abnormalities in the two species according to sex are shown in Figure 3, and in Figure 4 only for karyolitic cells due to a different frequency scale.

The body weight of sampled animals (Table 1) differed according to age classes but not for sex in both species (squirrels age: t=7.3, p <0.001, sex t=1.06, p=0.059; wild boar age: U=381, p <0.001, sex U=250, p=0.222)

Model selections and results of GLMs for each species and for every abnormality are reported in Supplemental materials Table S2. Models indicate no effects of the covariates on abnormalities frequencies, except for a moderate evidence of a positive effect of weight on the frequency of broken eggs (estimate ±SE = 0.009±0.003; p= 0.024) and a stronger evidence of a similar effect on the frequency of karyolitic cells in grey squirrels (estimate ±SE = 0.002± 0.001; p= 0.003); in wild boars the frequency of condensed chromatin was slightly higher in males (estimate ±SE = 1.452±0.627; p= 0.021). The stepwise backward procedure confirmed these results.

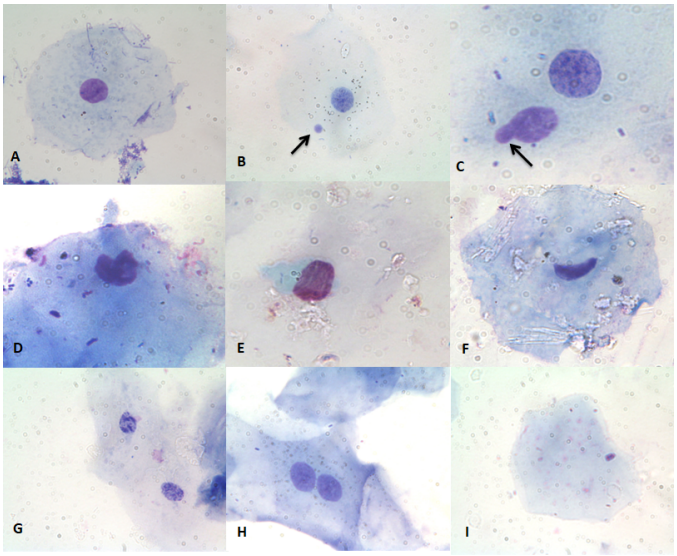

Figure 2 – Photomicrographs of exfoliated cells of the buccal mucosa of squirrel, stained with Giemsa and magnified 1000×. (A) typical cell; (B) cell with micronucleus (arrow); (C) nuclear bud (arrow); (D) Kidney-shaped nucleus; (E) broken eggs; (F) pycnotic nucleus; (G) nucleus with condensed chromatin; (H) binucleated cell and (I) karyolitic cells.

Table 1 – Sampled animals according to species and sex and their mean body weight.

Age class or sex	Sample size	Weight (±SD)
Sciurus carolinensis		
Juveniles	8	377±66 g
Adults	29	544±54 g
Females	22	527±94 g
Males	15	480±74 g
Sus scrofa		
Juveniles	16	12.5±16.0 kg
Adults	25	60.3±15.4 kg
Females	17	46.2±30.3 kg
Males	24	38.2±24.5 kg

Discussion

Management activities, such as controlling overabundant species, common in ungulates, and medium to large invasive alien species, offer the opportunity to handle freshly euthanised or culled animals (e.g. Santicchia et al., 2018, 2020). These control programs are often used to sample animals for many research topics, e.g. morphometry, reproductive success, presence of parasites, and animal health (e.g. Badou et al., 2021; Macchioni et al., 2022). We showed that controlled populations could also be used for research on genomic damages. Animals euthanised during control plans or research activities, such as the introduced grey squirrels, are generally inspected to collect routine data on sex, age and morphometry. During this inspection, buccal cells could be easily sampled. On the other hand, when animals are culled, blood in the mouth due to the shot may make some samples unsuitable for cell sampling. In our experience, about 60 % of collected wild boar samples were used to observe the required 1,000 cells with well-preserved cytoplasm. However, the quality of the samples decreases rapidly after shooting due to bacterial infection and cell lysis; therefore, it is fundamental to collect exfoliated buccal mucosa cells a few minutes after

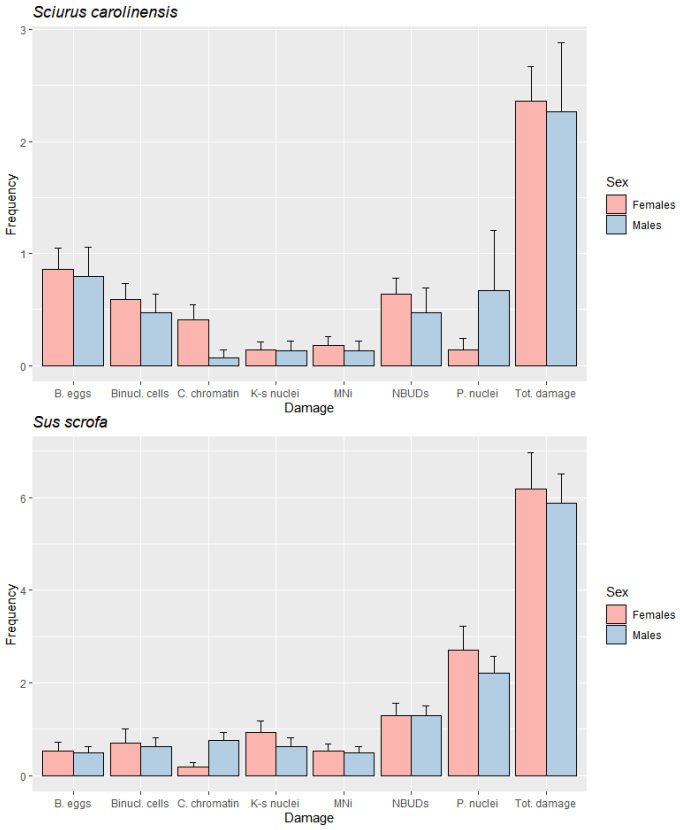

Figure 3 – Frequency of genomic damages (mean ± SE damage counted in 1,000 cells for every individual) in grey squirrel (above) and wild boar (below) according to sex. Abbreviations: B. eggs = Broken eggs, Binucl. Cells = Binucleated cells, C. chromatin = Condensed chromatin, K-s nuclei = kidney-shaped nuclei, Mni = Micronuclei, NBUDs = Nuclear buds, P. nuclei = Pyknotic nuclei.

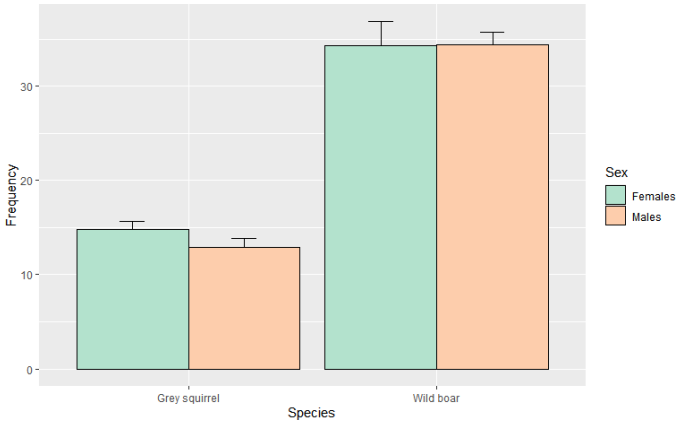

Figure 4 – Frequency of Karyolitic cells (mean ± SE damage counted in 1000 cells for every individual) in grey squirrel and wild boar according to sex.

the animal's death. Hunting activity and control plans regard only a limited number of species. For this reason, testing new procedures to sample buccal cells from live animals temporarily captured or otherwise immobilised will be essential.

It is known that some endogenous factors, such as age, gender and body weight, may modulate the level of genomic damage (Santovito et al., 2020b). In contrast with data obtained from most of the human populations (Santovito and Gendusa, 2020) and from bats (Benvindo-Souz et al., 2019a,b), sex and age did not seem to influence the level of DNA and nuclear damage in our samples. This lack of association may be related to the short life spans of squirrels and the fact that most of the sampled wild boars were less than two years old. We did not find any effect of body weight on the frequency of most damage, including MNi and NBUDs, supporting earlier results obtained by Benvindo-Souz et al. (2019b) and by Assunção (2016), who failed to observe a significant correlation between the levels of observed DNA damage and the body weight of the animals. A few effects found on broken eggs, karyolitic cells, and condensed chromatin, need further investigation and a better understanding of the possible association between the frequency of these abnormalities and animal health. In any case, research on wild animals is minimal, and the effects of intraindividual characteristics on the level of genomic damage need further investigation with larger sample sizes. In particular, comparing data of physiological stress, using faecal glucocorticoid metabolites as an integrated measure of plasma glucocorticoids (e.g. Santicchia et al., 2018, 2022a; Tranquillo et al., 2022), and genomic damage might reveal patterns of individual or population-level associations useful for population management or conservation.

In our laboratory procedure, pellets obtained by centrifugation were dissolved in a minimal amount of fixative and seeded on the slides to detect nuclear abnormalities by conventional staining with Giemsa. Alternatively, cells can be stained by Feulgen-Fast Green (FFG), Acridine Orange, DAPI (4,6-diamidino-2-phenylindole) Hoechst or PI (propidium iodide). For the laboratory protocols of these staining methods, see reference publications: Thomas et al., 2008; Cao et al., 2002; Sanchez-Siles et al., 2011; Casartelli et al., 1997; Surrallés et al., 1997. Feulgen-Fast Green is the staining method favoured by many researchers because of its DNA specificity and because cytoplasm appears transparent, enabling easy identification of MNi (Holland et al., 2008). MNi stained with FFG may be examined under both light or fluorescent microscopy. However, the shortcoming of this staining method is that it is relatively lengthy and may lead to the underscoring of MNi (see Holland et al., 2008 for a review). In contrast, the shortcoming of the other stain methods alternative to Giemsa is that the fluorescence is not permanent but fades with time.

The application of BMCyt assay to wild animal populations opens up many research opportunities and the possibility of comparing populations living in areas with different degrees of anthropic disturbance and alteration of environmental quality. This assay is relatively rapid and low cost and offers the opportunity to analyse many cells (Benvindo-Souz et al., 2019a,b) in comparison with other methods that require blood samples or tissue biopsy. Moreover, our developed protocol readily applies in the field since it does not require special equipment. Buccal cells were collected from the inner side of the lower lip and palate using toothbrushes of different sizes. Collected cell samples maintained in a fixative solution could then be stored at room temperature before the analysis. For these reasons, BMCyt assay is widely used in humans to evaluate DNA damage and chromosomal instability due to acute or chronic exposure to environmental xenobiotics (Cobanoglu et al., 2019; Dos Santos et al., 2022; Nagaraju et al., 2022; Santos et al., 2023). However, its application to wild animals is recent, and studies are still limited (e.g. Benvindo-Souz et al., 2019a,b). MNi and NBUDs represent biomarkers of DNA damage usually associated with the presence of genotoxic agents in the environment, and BMCyt assay can detect this damage before the development of clinical symptoms (Stich et al., 1984).

We also observed the presence of pyknotic, karyorrhetic and condensed chromatin nuclei, which are associated with apoptotic processes, as well as binucleated cells that represent the result of damage to the cytokinesis mechanism as consequence of cell cycle arrest due to chromosomes malsegregation or telomere dysfunction (Bolognesi et al., 2013)

In this work, we applied the BMCyt assay to wild grey squirrel and wild boar populations living in habitats with different anthropogenic pressure. Squirrels and wild boars show a great diversity of diets and, inhabiting both natural and anthropized environments, they can enter in contact with potentially contaminated food and with environmental pollutants (Cobanoglu et al., 2019; Panico et al., 2020; Acito et al., 2022; Dos Santos et al., 2022). In this context, the buccal MN assay provides an important tool for analysing the genotoxic effects of environmental xenobiotics, also considering that the oral mucosa is in constant contact with environmental agents during feeding, represent-

ing an important target for toxic substances that have been ingested or inhaled (Rosa et al., 2013).

As there are no reference values beyond which the level of cell damage can be considered harmful and reduce the fitness of individuals, it is essential to use this method to compare populations living in environments with different anthropogenic pressures. For example, comparing populations living in urban areas or with intensive farming with reference populations living in natural environments can facilitate to understand the effects of stressors associated with a growing human footprint. At the individual level, recording fitness related parameters, such as body weight, reproductive condition, age, as a proxy for survival, and glucocorticoid metabolites (Santicchia et al., 2022a,b) will increase insight into the natural and stress-induced variation in the amount of cell damage.

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Supplemental information

Additional Supplemental Information may be found in the online version of this article:

Table S1 Model selection.

Table S2 Full poisson or negative binomial GLM results.