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Air pollution, but not physiological stress, is associated with genomic damage of invasive grey squirrels from urban and agricultural areas

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A R T I C L E I N F O

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A B S T R A C T

Anthropogenic disturbances, such as habitat fragmentation and pollutants, can act as stressors limiting wildlife persistence and adaptation, and mammals are particularly vulnerable to airborne pollutants and pesticides that disrupt glucocorticoid (GC) signalling. The synergic impact of pollution and chronic GCs exposure may cause genomic damage reducing genetic diversity and adaptative potential. Using the invasive Eastern grey squirrel (*Sciurus carolinensis*), we assessed genomic damage, micronuclei (MNi) and nuclear buds (NBUDs), along a gradient from urban centres to agricultural areas, with increasing air pollution, and related this to faecal glucocorticoid metabolites (FGMs). Genomic damage varied significantly among study areas, with lower MNi and NBUDs in a hilly urban area. In the central urban area, MNi frequency was lower than in the agricultural areas. Conversely, in the peripheral urban area, near major roadways, MNi and NBUDs were comparable to those in agricultural areas, suggesting a similar exposure to genotoxic stressors, likely originating from different sources. A curvilinear relationship was observed between air pollutants and genomic damage: MNi and NBUDs levels remained stable at low pollution levels and decreased as pollution increased. Sex, body condition and age had no significant effects. FGMs did not significantly influence MNi or NBUDs across urban and agricultural areas. Our results show that squirrels in a hilly urban area had less genomic damage than those in high-traffic peripheral or agricultural areas. These findings highlight the importance of urban planning that incorporates refuges capable of mitigating environmental stressors and strategies that limit genotoxic effects in urban and agricultural areas.

1. Introduction

The rapid expansion of human activities and urbanisation presents both opportunities and significant challenges for wildlife (Elmqvist et al., 2013). Urban and semi-urban environments can offer novel resources such as stable food supplies, shelter, and reduced predation risk for adaptable species (Fischer et al., 2012; Shochat, 2004). As a result, some mammals, particularly generalists, have successfully colonised urban areas and even altered their behaviour and activity patterns to thrive in these human-dominated landscapes (Bateman and Fleming, 2012; Ritzel and Gallo, 2020). However, for many other species, these rapidly changing environments pose substantial ecological and physiological constraints. Fragmentation of natural habitats, increased exposure to pollutants, artificial light and noise, altered predator-prey dynamics, and competition with invasive or synanthropic species can all limit the ability of native mammals to persist in or adapt to such settings (Rega-Brodsky et al., 2023; Villaseñor et al., 2014).

Environmental pollution has emerged as one of the most pervasive

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and complex forms of anthropogenic impact on terrestrial mammals. Pollution manifests in multiple forms, including chemical contaminants (such as pesticides, heavy metals, and industrial byproducts), as well as air, light, and noise pollution. These stressors affect mammals in diverse and often synergistic ways, disrupting physiological functions, altering behaviour, and reducing overall fitness (Aulsebrook et al., 2020). Air pollution, in particular, poses significant and growing risks to terrestrial mammals (Ogwu et al., 2024). Fine particulate matter, ozone, nitrogen oxides, and other airborne pollutants can infiltrate deep into the respiratory tract, leading to inflammation, respiratory distress, oxidative stress, and cardiovascular dysfunction. These effects are especially pronounced in urban wildlife populations, which are chronically exposed to elevated levels of pollution (Isaksson, 2010).

The relationship between air pollution and genomic damage in terrestrial wildlife has become a critical focus in ecotoxicology and wildlife conservation. Terrestrial species exposed to air pollutants in both anthropogenic and natural habitats are particularly susceptible to genomic damage. Airborne contaminants such as polycyclic aromatic hydrocarbons, heavy metals, volatile organic compounds, and particulate matter have been shown to induce oxidative stress, inflammation, and DNA damage (Møller et al., 2014; Valverde and Rojas, 2009). Also, pesticides are known for their genotoxicity in mammalian systems, inducing high frequencies of micronuclei, DNA strand breaks, chromosomal aberrations, and oxidative DNA damage (Bolognesi, 2003; Das et al., 2007; Quinn-Hosey et al., 2012). So far, few studies have investigated whether the relationships between environmental pollutants and DNA damage differ between populations of mammals in urban and agricultural areas, and studies on terrestrial mammals are lacking (Keilen et al., 2022; Pepey et al., 2025; Weijs et al., 2019). Moreover, studies on free-ranging vertebrates document that environmental exposures modify also thyroid hormones responses, which synergically interact with glucocorticoid hormones, ultimately impacting populations viability (Rolland, 2000). In human studies, chronic exposure to elevated glucocorticoids (GCs) has been associated with increased DNA and RNA oxidation, suggesting a mechanistic link between high glucocorticoid levels and genomic instability (e.g. DNA damage including strand breaks and micronuclei; Joergensen et al., 2011).

One widely used biomarker of genomic damage is the formation of micronuclei (MNi), which are small, extranuclear bodies that originate from chromosomal fragments or whole chromosomes that fail to be incorporated into the nucleus after cell division (Fenech et al., 2011). In addition to MNi, chromosomal instability can also be assessed through the presence of nuclear buds (NBUDs), which are indicators of the active elimination of amplified DNA or excess chromosomes from aneuploid or genomically unstable cells (Bolognesi et al., 2013). The formation of micronuclei in terrestrial wild species has significant ecological implications. Genomic damage can impair reproductive success, reduce genetic diversity, and compromise a population's capacity to adapt to environmental changes (Bickham et al., 2000). To monitor such risks, species that serve as ecological indicators or sentinel species are often employed to assess the genotoxicity of polluted environments. For example, small mammals are frequently used in biomonitoring studies due to their high sensitivity to environmental pollutants and their ecological relevance (Ma et al., 2023). These species often occupy key positions in food webs and have relatively small home ranges, making them reliable indicators of local environmental conditions. Moreover, small mammals can accumulate substantial quantities of pollutants from their surroundings through diet, inhalation, and dermal absorption, which makes them excellent bioindicators of environmental quality (Ma et al., 2023; Petkovšek et al., 2014).

Our research aimed to investigate the influence of environmental stressors on genomic damage of a terrestrial mammal species inhabiting anthropized environments (urban and agricultural areas). Most current literature addresses this relationship primarily in sentinel or model species and there is a lack of longitudinal field studies on wildlife (Claro et al., 2024). We selected the introduced Eastern grey squirrel (*Sciurus carolinensis*) as a model species, building on ongoing research examining the adaptation of both native and invasive squirrels across a gradient of anthropogenic habitats (Santicchia et al., 2024; Tranquillo et al., 2024).

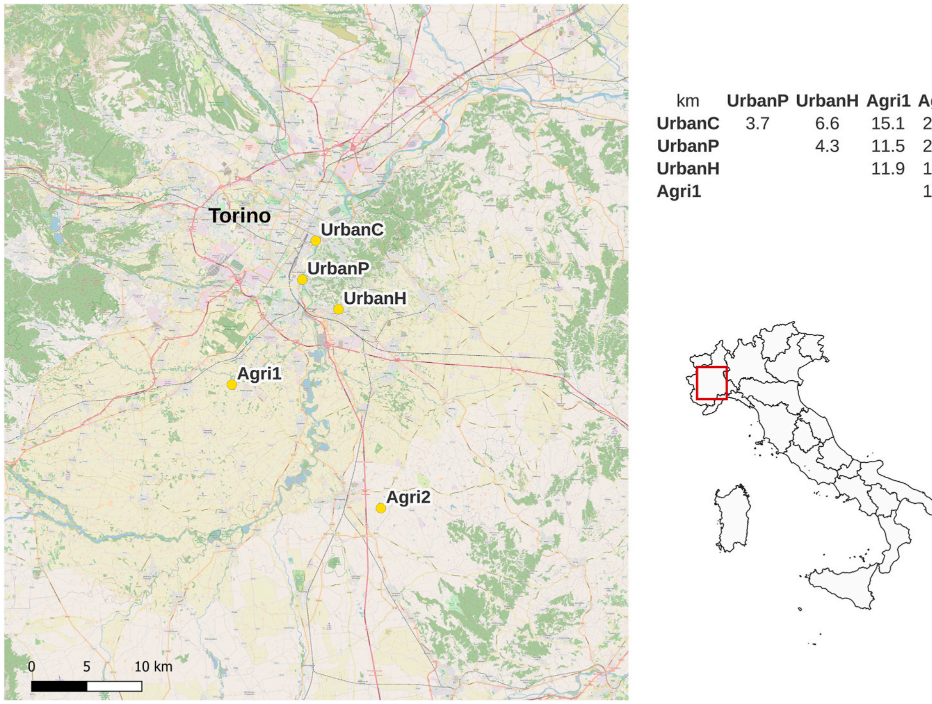
The micronucleus test in exfoliated buccal mucosa cells is a widely used non-invasive biomarker of genotoxicity, effectively detecting DNA damage caused by pollutants such as atmospheric contaminants (Panico et al., 2020), dust particles (Wultsch et al., 2019), and pesticides (Dos Santos et al., 2022). This method has also been successfully applied to wild (Benvindo-Souz et al., 2019; Bertolino et al., 2023) and domestic (Santovito et al., 2022) mammals confirming its reliability across species and environmental contexts. Similarly, the measurement of faecal glucocorticoid metabolites (FGMs) is a commonly used non-invasive technique to assess physiological stress across various taxa and provide insight into how environmental and anthropogenic stressors impact individuals and populations (Breuner et al., 2013; Santicchia et al., 2022a, 2022b, 2024). In wildlife, a prolonged elevation in glucocorticoid levels and/or frequent exposure to stressors, termed as "chronic stress", is assessed through FGMs, an integrated measure of both baseline and stress-induced GC levels, proxy of an animal's hypothalamic–pituitary–adrenal axis activity over a specific period of time (Palme, 2019).

To better evaluate the outcome of environmental stressors on grey squirrels we explored effects on genomic damage: 1) across a gradient of study areas, from urban centres to agricultural areas, to capture the potential influence of different sources of anthropogenic impact; 2) using air pollution data, which provided an additional layer of environmental context; 3) using FGM levels, in terms of chronic exposure to environmental stressors. Intensively farmed environments are known for their high levels of chemical pollutants commonly associated with agricultural practices (Carvalho, 2017; Zhou et al., 2025). In contrast, policies implemented in European cities over the past decades have aimed to reduce pollution levels in urban centres (Jonidi Jafari et al., 2021; Viana et al., 2020). For these reasons, we predicted that squirrel populations exposed to intensive agricultural activities would exhibit higher levels of genomic damage (MNi and NBUDs) compared to those living in urban areas. Specifically concerning air pollution, and its effect on genomic damage already reported for animal models and humans (Møller et al., 2014; Valverde and Rojas, 2009), we predicted that MNi and NBUDs would increase following a rise in air pollution levels. Finally, we predicted that MNi and NBUDs would increase at elevated FGM levels and at higher levels of anthropogenic disturbance. Indeed, chronic exposure to elevated GCs is associated with oxidative stress, chromosomal instability and, ultimately may predispose individuals to genomic damage (Breuner et al., 2013; Joergensen et al., 2011), and this relationship might be mediated by differential pressures of anthropogenic stressors (e.g. low/high urbanisation, pollution).

2. Materials and methods

2.1. Study areas

The present study was conducted in five areas reflecting different levels of anthropogenic disturbance in and around the city of Turin, Piedmont, Northern Italy, between February 2021 and May 2023. The locations and relative distances of the study areas are shown in Fig. 1. Specifically, we trapped grey squirrels in three sites in the urban environment: an urban central (UrbanC) area (Orto Botanico, 5.2 ha), which is a botanical garden in the city centre, close to a main road, but where vehicle emissions are shielded by vegetation. The second site is an urban peripheral (UrbanP) area (ILO, 5.8 ha) which is a private park on the outskirts of the city. This area is located near a highway and a major six-lane road. There is no vegetation to provide a shield and during peak traffic hours, the smell of vehicle exhaust fumes can be detected in the study area. The third site is an urban hilly (UrbanH) area (Moncalieri, 6.9 ha) which is a private garden on the hills surrounding the city in a green zone with low levels of urbanisation and 2 km far from the



km	UrbanP	UrbanH	Agri1	Agri2
UrbanC	3.7	6.6	15.1	24.9
UrbanP		4.3	11.5	21.9
UrbanH			11.9	18.5
Agri1				17.6

Fig. 1. Locations and relative distances (km) between the study areas. Stamen Terrain Labels shown as basemap.

highway which is located in the plain at the foot of the hills. We also captured grey squirrels in two study areas in the agricultural environment: Piobesi (Agri1, 5.8 ha) 1.5 km far from the nearest village (Piobesi Torinese) and Commande (Agri2, 4.5 ha) 3 km far from the nearest village (Carmagnola) and 1.3 km far from a nearby major road, both sites are private parks surrounded by intensively cultivated fields with crop rotation. Characterisation of the study areas landscape is shown in Fig. 2.

2.2. *Trapping and sampling*

Trapping was carried out in each study area with two to four capture-removal sessions, each lasting two to five days. Captured grey squirrels were culled in accordance with European Regulation 1143/2014. We used single-capture live traps (Model 202, Tomahawk Live Trap Co., Hazelhurst, WI, USA), more or less homogeneously distributed in each study area, with distances of 10–50 m between traps. The number of traps varied for each study area (15 in UrbanC; 15 in UrbanP; 14 in

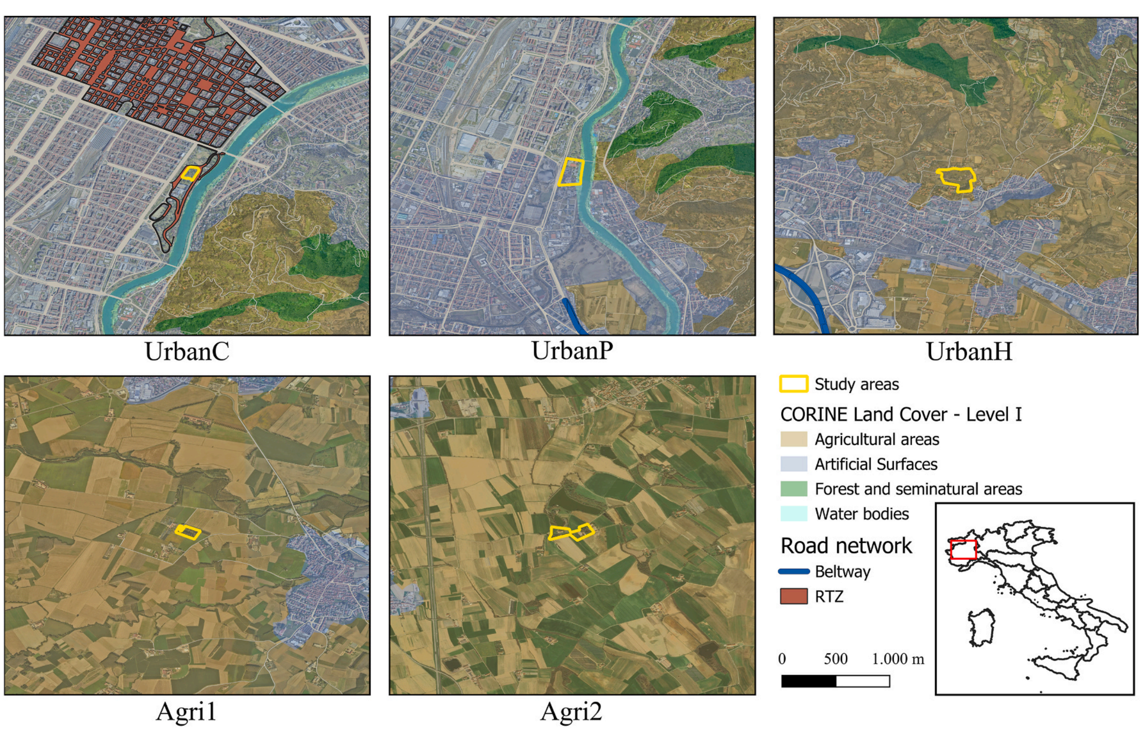


Fig. 2. Characterisation of urban and agricultural study areas. RTZ: restricted traffic zone.

UrbanH; 15 in Agri1; 23 in Agri2). Traps were placed on the ground, with a fine mesh added underneath to limit contamination from urine and faeces. They were baited with hazelnuts and checked three times daily to reduce confinement time and minimise the interval between defecation and sample collection. Upon capture, each squirrel was euthanised using CO_2 inhalation and weighed to the nearest 5 g with a spring balance (Pesola AG, Baar, Switzerland). The length of the right hind foot was measured with a thin ruler to the nearest 0.5 mm. The sex and reproductive condition of the squirrels were determined through the inspection of external genitalia and nipples, following the methods described in Dantzer et al. (2016) and Santicchia et al. (2018).

The buccal assay was performed as described by Bertolino et al. (2023), with minor modifications. We sampled 161 squirrels, comprising 85 females and 76 males. One sample was excluded as an outlier from the MNi dataset. The sample sizes for each study area, for both MNi and NBUDs, were as follows: UrbanC = 29; UrbanP = 27 (MNi), 28(NBUDs); UrbanH = 46; Agri1 = 42; Agri2 = 16). Briefly, exfoliated buccal mucosa cells were collected from fresh carcasses by scraping the inner lining of one or both cheeks with a small toothbrush for at least 1 min per side, ensuring the retrieval of an adequate number of epithelial cells from distinct areas of the oral mucosa. The toothbrush tip was then immersed in a fixative solution of methanol and acetic acid (3:1), shaken for 1 min, and stored at 4 °C prior to analysis. After carcass sampling, faecal samples were collected from underneath the traps using forceps and placed individually into 1.5-ml vials (Dantzer et al., 2016). The trap, mesh and the ground under the trap were cleaned to remove possible remains of faecal material. We placed faecal samples into an insulated bag with wet ice packs while in the field and then stored them at −20 °C within 3–4 h after collection (Dantzer et al., 2016).

Ethical statement

Trapping and handling of squirrels were carried out in accordance with the Guidelines for the treatment of animals in behavioural research and teaching (Animal Behaviour, 2020, 159, I–XI; https://doi.org/10.1016/j.anbehav.2019.11.002). The removal of grey squirrels was mandatory following European Regulation 1143/2014. Grey squirrels were euthanised by CO_2 inhalation following international animal welfare guidelines (Close et al., 1996, 1997; Leary et al., 2013). Approval and legal requirements according to the Italian Wildlife Protection and Hunting Law L.N. 157 from 1992 and authorisations N. DD 1015 of 20/03/2020 and N. DD 1677 of 30/03/2023 from Città Metropolitana di Torino.

2.3. Buccal micronucleus cytome (BMCyt) assay

In the lab, cells were collected by centrifugation, the supernatant discarded, and the resulting pellet resuspended in a minimal volume of fixative. This suspension was spread onto slides using conventional staining with 5% Giemsa (pH 6.8), prepared in Sörensen buffer (comprising 50% Na_2HPO_4 and 50% KH_2PO_4).

Microscopic analysis was performed at 1000 × magnification using a light microscope. Following established criteria, MNi and NBUDs were scored in 1000 cells with well-preserved cytoplasm per subject (Thomas et al., 2009). Cytogenetic scoring was conducted under blinded conditions by two expert reviewers. All observed cases of genomic damage (MNi and NBUDs) detected by microscopy were photographed and analysed collectively using computer software.

2.4. Air pollution variables

Air pollution data were kindly supplied by the Regional Agency for Environmental Protection (ARPA – Agenzia Regionale per la Protezione Ambientale) of Piedmont Region, which systematically monitors urban pollutants such as nitrogen oxides and particulate matter and provides annual assessments of air quality across the Turin metropolitan area (ARPA, 2021, 2022, 2023, 2024). The raw dataset (in NetCDF COARDS format) covered $PM_{2.5}$, PM_{10} and NO_2 daily mean concentrations, and O_3 maximum daily concentration (on an 8-h moving average), with a 4 × 4 km spatial resolution for the period 2020-2023. All values were referred at a standard elevation of 10 m above ground level. According to the locations of the traps where each individual was caught, a data extraction procedure — developed in R/Rstudio using the ncdf4 package (Pierce, 2024) — calculated from the raw data, for all the variables, the average value for the three months preceding the capture.

2.5. Extraction and quantification of FGMs

We analysed single faecal samples from a subset of 85 grey squirrels (41 males and 44 females). One sample was excluded from the subset used in the models including MNi due to an outlier in the MNi values. The FGMs sample sizes for each study area were as follows: UrbanC = 23; UrbanP = 18(subset for MNi model), 19(subset for NBUDs model); UrbanH = 16; Agri1 = 15; Agri2 = 12. Samples were oven-dried (80 °C) overnight, ground up under liquid nitrogen, homogenised and weighed to 0.050 g (±0.004 g), and extracted using 80% methanol (1 ml), shaking at 1500 r.p.m on a multivortex for 30 min, centrifuging at 2500 g for 15 min. An aliquot of the supernatant was diluted in assay buffer (1 + 9) and assayed using a 5α-pregnane-3β, 11β, 21-triol-20-one enzyme immunoassay (EIA) to measure FGM concentrations (ng g−1 dry faeces; Romeo et al., 2020; Santicchia et al., 2018; Touma et al., 2003). This in-house EIA detects GC metabolites with a 5α-3β, 11β-diol structure (for cross-reactivity see Touma et al., 2003). Methods of EIA validation for Eastern grey squirrels can be found in detail elsewhere (Bosson et al., 2013). Samples were analysed in duplicate. Intra- and inter-assay CVs were 2.2% and 3.3%, respectively.

2.6. Statistical analyses

To test our predictions, we used generalised linear mixed-effects models (GLMM) with two approaches: 1) to test the DNA damage we used the number of micronuclei (MNi) in 1000 cells as dependent variable, while 2) to test the chromosomal instability we used the number of nuclear buds (NBUDs) in 1000 cells as dependent variable. Since data were collected in more than one year, the year was included as a random intercept term. We assessed dispersion parameter and applied a Conway-Maxwell-Poisson distribution for underdispersion in all models. For both approaches, GLMMs included sex, age (juveniles-subadults or adults) and study area as fixed effects, while body condition (the score of the second component from a PCA of body mass and foot length, loadings: 0.707 body mass – 0.707 foot length; Tranquillo et al., 2022) was included as continuous explanatory variable. Two age classes were defined using a combination of external genitalia and, for females, body mass: 1) juveniles-subadults: males with abdominal testes and a scrotum not yet evident or very small and without dark staining, and nonbreeding females (anestrous, vulva small, no longitudinal opening, not lactating) with body mass <460 g; 2); adults: males with abdominal testes but an evident scrotum, semiscrotal and scrotal testes, and nonbreeding, postestrous, or lactating females with body mass≥460 g.

Due to high correlation among NO_2, O_3, $PM_{2.5}$ and PM_{10} (see Table S1) a PCA was applied in order to define a unique variable including all air pollutants. The PC1 (loadings: 0.45 NO_2 + 0.43 O_3 + 0.55 $PM_{2.5}$ + 0.56 PM_{10}, eigenvalue 3.00) explained 75% of the total variance and can be considered as a unique variable with high scores for all four air pollutants. To consider possible nonlinear (curvilinear) effects of air pollutants (PC1) on MNi or NBUDs, its second-order orthogonal polynomial effect was included in the models. High multicollinearity of polynomial PC1 and study area (VIF >20, Zuur et al., 2010) prevented us to include polynomial PC1 as a fixed effect in the two models described above. Thus, to investigate the effects of air pollutants, we performed a set of separate models (one model on MNi and one on NBUDs) using the same model structure including polynomial

PC1 while excluding study area fixed effect, in order to test for possible influences of air pollutants on genomic damage and chromosomal instability.

Subsequently, for both MNi and NBUDs, we compared a full model including study area (MNi/NBUDs ~ sex + age + body condition + study area) with a full model including polynomial PC1 (MNi/NBUDs ~ sex + age + body condition + polynomial PC1) using the Akaike's information criterion (AICc scores). For both MNi and NBUDs, the best model structure (lowest AICc and delta AICc >2) was the one with study area as fixed effect. Therefore, we used this model structure to run models including FGM concentrations (transformed using the natural logarithm, ln of ng/g dry faeces) and its interaction with study area to test for potential effect of physiological stress on MNi and NBUDs depending on degree of anthropogenic disturbance (study area). The year was included as a random intercept term. These models testing for an effect of FGMs were run using a subset (n = 84 for MNi, 1 outlier excluded; n = 85 for NBUDs) of our data. Due to the relatively small sample size in the subset and to avoid overfitting, in these models with FGM concentrations we included only significant variables derived from the previous models on the full dataset (MNi/NBUDs ~ lnFGMs + study area + lnFGMs*study area).

Model selection, where applicable (e.g. models on the full dataset), was performed through stepwise backward elimination of non-significant parameters (Lewis et al., 2011) and without eliminating those variables specifically related to our predictions. Where necessary, comparisons of factors with more than two levels were carried out with Tukey test for multiple comparisons. Residuals were visually inspected to verify the assumptions of normality and homoscedasticity (Zuur et al., 2010). GLMM were performed using package "glmmTMB" (Brooks et al., 2017). All the statistical analyses were performed in R (R Core Team, 2023).

3. Results

3.1. Differences among study areas in MNi and NBUDs

The mean MNi ±SE was 0.56 ± 0.06 (range: 0–3), while the mean NBUDs ± SE was 1.21 ± 0.10 (range: 0–5). The final GLMM model revealed significant differences in MNi (Fig. 3) and NBUDs (Fig. 4) counts among study areas (Table 1). There was no effect of sex, age, or body condition on either MNi or NBUDs (Table S2), and these variables were removed during model selection. Pairwise comparisons revealed lower MNi values in the hilly (UrbanH) area compared with the peripheral urban area (UrbanP) and with the two areas with intensive agriculture (Agri1, Agri2), while the peripheral urban area did not differ from the agricultural areas. MNi values were lower in the central (UrbanC) area compared to the agricultural areas (Table 1, Fig. 3). The NBUDs in the hilly area were similar to those in the central urban area but were again lower than those in the peripheral urban area and the agricultural areas (Table 1, Fig. 4). No significant differences were found between the central urban area and the other areas.

3.2. Effect of air pollutants (PC1) on MNi and NBUDs

The final model identified a significant curvilinear influence of air pollutants (PC1 of NO_2, O_3, $PM_{2.5}$ and PM_{10}) both on MNi (Table 2) and NBUDs. Specifically, MNi (Fig. 5) and NBUDs (Fig. 6) of grey squirrels at low levels of air pollution (PC1) were initially stable or slightly increasing, but as air pollution increased, MNi and NBUDs declined. There was no effect of sex, body condition and age (Table S3) thus these variables were removed during model selection.

3.3. Faecal glucocorticoid metabolites (FGMs), MNi and NBUDs

Models testing the effect of physiological stress, in relation to

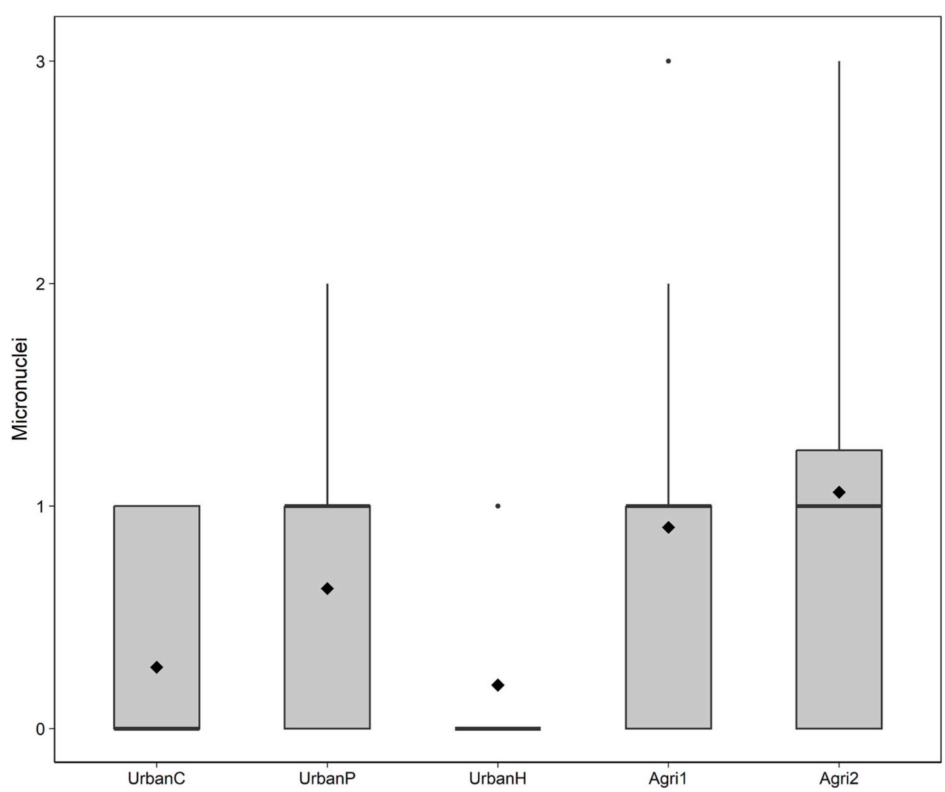


Fig. 3. Micronuclei (MNi) counts for grey squirrel samples (n = 160) across five study areas: UrbanC (urban central area, n = 29), UrbanP (urban peripheral area, n = 27), UrbanH (urban hilly area, n = 46), Agri1 (agricultural area, n = 42) and Agri2 (agricultural area, n = 16). The boxplots display the median (solid horizontal line), mean (black diamond), and the interquartile range (1st [25%] and 3rd [75%] quartiles).

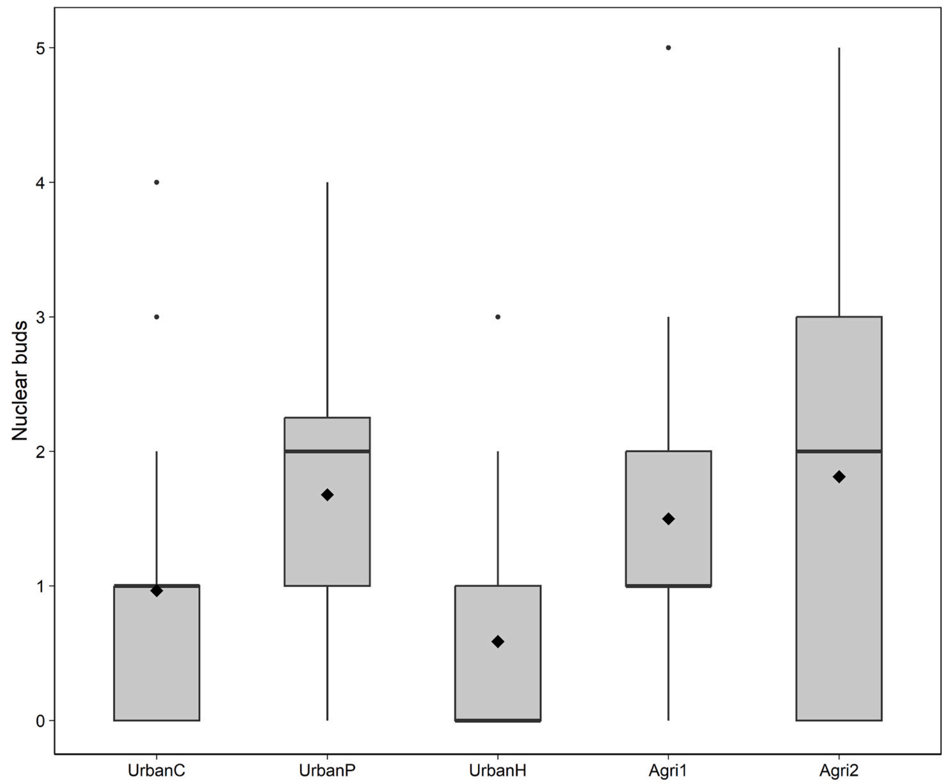


Fig. 4. Nuclear buds (NBUDs) count for grey squirrel samples (n = 160) across five study areas: UrbanC (urban central area, n = 29), UrbanP (urban peripheral area, n = 28), UrbanH (urban hilly area, n = 46), Agri1 (agricultural area, n = 42) and Agri2 (agricultural area, n = 16). The boxplots display the median (solid horizontal line), mean (black diamond), and the interquartile range (1st [25%] and 3rd [75%] quartiles).

Table 1
Final models examining the effect of study area on MNi (160 observations) and NBUDs (161 observations; see "Statistical analyses" section for details). Year was included as a random intercept. *P*-values are derived from Tukey's test for multiple comparisons.

Explanatory variables	Parameter estimate (±SE)	Z value	p
Micronuclei (MNi)			
UrbanC - Agri2	−1.35 ± 0.37	−3.62	0.003
UrbanP - Agri2	−0.52 ± 0.27	−1.93	0.29
UrbanH - Agri2	−1.69 ± 0.36	−4.66	<0.001
Agri1 - Agri2	−0.16 ± 0.22	−0.72	0.95
UrbanC - UrbanP	−0.83 ± 0.38	−2.16	0.18
UrbanH - UrbanP	−1.17 ± 0.37	−3.14	0.01
Agri1 - UrbanP	0.36 ± 0.24	1.53	0.53
UrbanC - UrbanH	0.34 ± 0.45	0.76	0.94
Agri1 - UrbanH	1.53 ± 0.34	4.53	<0.001
Agri1 - UrbanC	1.19 ± 0.35	3.41	0.005
Nuclear buds (NBUDs)			
UrbanC - Agri2	−0.63 ± 0.27	−2.36	0.13
UrbanP - Agri2	−0.08 ± 0.24	−0.32	1.00
UrbanH - Agri2	−1.13 ± 0.27	−4.18	<0.001
Agri1 - Agri2	−0.19 ± 0.23	−0.84	0.92
UrbanC - UrbanP	−0.55 ± 0.24	−2.30	0.14
UrbanH - UrbanP	−1.05 ± 0.24	−4.32	<0.001
Agri1 - UrbanP	−0.11 ± 0.20	−0.58	0.98
UrbanC - UrbanH	0.50 ± 0.27	1.83	0.35
Agri1 - UrbanH	0.94 ± 0.23	4.05	<0.001
Agri1 - UrbanC	0.44 ± 0.23	1.92	0.30

Table 2
Final models including second-order orthogonal polynomial effect of air pollutants (PC1 of NO_2, O_3, $PM_{2.5}$ and PM_{10}) on MNi (160 observations) and NBUDs (161 observations; see "Statistical analyses" section for details). Year was included as a random intercept.

Explanatory variables	Parameter estimate (±SE)	χ^2 degrees of freedom	p
Micronuclei (MNi)			
PC1	−4.23 ± 1.54		
$PC1^2$	−2.76 ± 1.41	$\chi^2 = 9.08$; df = 2	0.01
Nuclear buds (NBUDs)			
PC1	−3.71 ± 1.11		
$PC1^2$	−1.55 ± 1.03	$\chi^2 = 11.74$; df = 2	0.003

anthropogenic disturbance, on MNi and NBUDs were conducted on a subset of 85 samples (with one sample excluded as an outlier in the MNi dataset) for which FGM levels were measured. The models included study area (the only significant variable in the models on the full dataset), FGMs and their interaction. For both models on MNi and NBUDs (Table 3), there was no effect of FGMs (MNi model: $\chi^2 = 2.51$, df = 1, p = 0.11; NBUDs model: $\chi^2 = 3.05$, df = 1, p = 0.08), study area (MNi model: $\chi^2 = 5.83$, df = 4, p = 0.21; NBUDs model: $\chi^2 = 6.33$, df = 4, p = 0.18) or their interaction (MNi model: $\chi^2 = 5.24$, df = 4, p = 0.26; NBUDs model: $\chi^2 = 6.00$, df = 4, p = 0.20; not excluded from the model since specifically related to our prediction). However, there was a weak difference of the relationship of FGMs with NBUDs between study area urban peripheral (UrbanP) and agricultural (Agri2) (estimate ± SE = 1.14 ± 0.47, z value = 2.42, p = 0.02, Table 3; for all contrasts see Table S4).

4. Discussion

In our research, we used micronuclei (MNi) and nuclear buds (NBUDs) as biomarkers to evaluate the impact of urbanisation and environmental pollutants on grey squirrel populations inhabiting a large city and intensive agricultural areas. Within the urban environment, the level of genomic damage varied by location, whereas levels appeared more uniform across agricultural areas. MNi and NBUDs were lower in hilly urban area (UrbanH). In contrast, the peripheral urban area

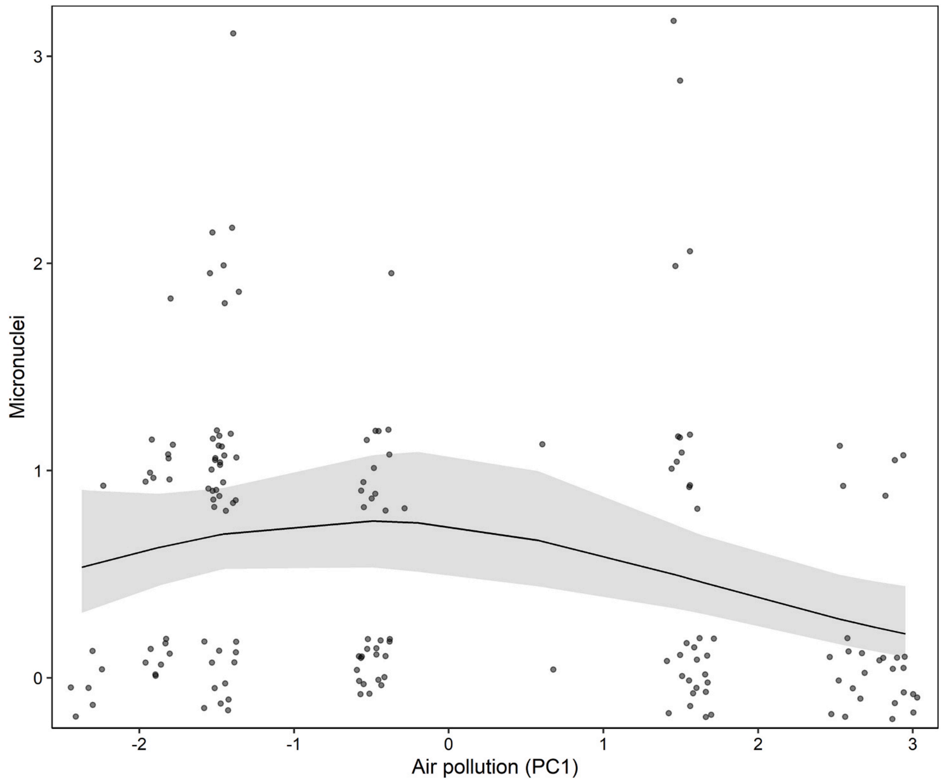


Fig. 5. Relationship between micronuclei (MNi) and air pollution (PC1 of NO_2, O_3, $PM_{2.5}$ and PM_{10}) in grey squirrels (n = 160). The bold line represents the predicted relationship, while the shaded areas indicate the 95% confidence intervals. Symbols represent observed values, with full circles jittered to prevent overlap.

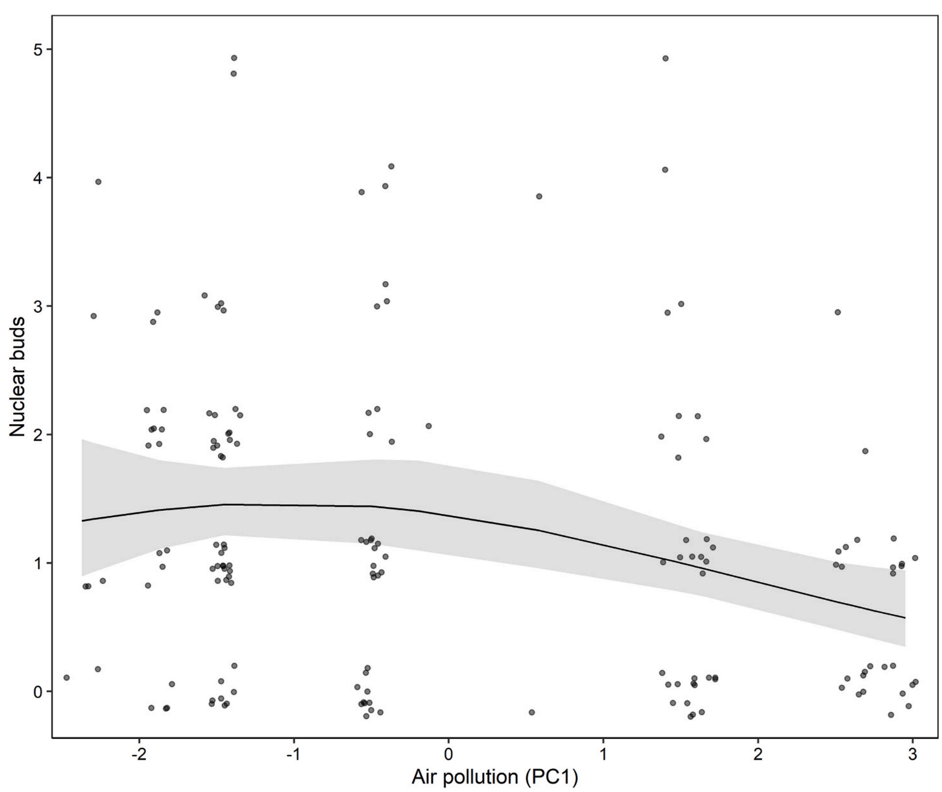


Fig. 6. Relationship between nuclear buds (NBUDs) and air pollution (PC1 of NO_2, O_3, $PM_{2.5}$ and PM_{10}) in grey squirrels (n = 161). The bold line represents the predicted relationship, while the shaded areas indicate the 95% confidence intervals. Symbols represent observed values, with full circles jittered to prevent overlap.

(UrbanP) – located next to a major road with heavy traffic – exhibited levels of genomic damage similar to those observed in the two intensive

Table 3

Models including FGMs (transformed using the natural logarithm, ln of ng/g dry faeces) on MNi (84 observations) and NBUDs (85 observations; see the "Statistical analyses" section for details). Year included as a random intercept.

Explanatory variables	Parameter estimate (±SE)	Z value	p
Micronuclei (MNi)			
UrbanC[a]	−7.29 ± 4.45	−1.64	0.10
UrbanP[a]	0.39 ± 4.57	0.09	0.93
UrbanH[a]	−2.09 ± 7.24	−0.29	0.77
Agri1[a]	−7.81 ± 4.02	−1.94	0.05
lnFGM	−0.53 ± 0.33	−1.59	0.11
UrbanC * lnFGM[b]	0.81 ± 0.58	1.40	0.16
UrbanP * lnFGM[b]	−0.09 ± 0.60	−0.14	0.89
UrbanH * lnFGM[b]	0.02 ± 0.94	0.02	0.98
Agri1 * lnFGM[b]	1.00 ± 0.54	1.85	0.06
Nuclear buds (NBUDs)			
UrbanC[a]	−5.15 ± 3.76	−1.37	0.17
UrbanP[a]	−8.83 ± 3.62	−2.44	0.02
UrbanH[a]	−4.90 ± 3.72	−1.32	0.19
Agri1[a]	−6.10 ± 4.15	−1.47	0.14
lnFGM	−0.59 ± 0.34	−1.75	0.08
UrbanC * lnFGM[b]	0.58 ± 0.50	1.16	0.25
UrbanP * lnFGM[b]	1.14 ± 0.47	2.42	0.02
UrbanH * lnFGM[b]	0.56 ± 0.49	1.16	0.25
Agri1 * lnFGM[b]	0.78 ± 0.56	1.38	0.17

[a] Study area Agri2 held as reference level.

[b] Interaction of study area Agri2 by lnFGM held as reference level.

agricultural areas (Agri1, Agri2), suggesting comparable exposure to genotoxic stressors, albeit of different origins. These results were not influenced by the sex, body condition, or age of the animals, suggesting that the observed levels of genomic damage were primarily associated with environmental exposure rather than individual biological traits, reinforcing the interpretation that external environmental stressors, rather than intrinsic factors (Sandoval-Herrera et al., 2021; Santovito et al., 2022), are the main drivers of the genotoxic effects observed in the study populations.

In urban areas, pollutants are primarily linked to vehicular emissions, including nitrogen oxides, particulate matter, and polycyclic aromatic hydrocarbons. In contrast, in agricultural landscapes, exposure is mainly driven by agrochemicals such as pesticides, herbicides, and fertilisers. Despite these differences, both environments appear to impose a significant genotoxic burden on local wildlife. Living in urban environments can be challenging for squirrels, but the extent of these challenges largely depends on the specific area of the city they inhabit (Koprowski, 2005). The hills surrounding the city of Turin represent mainly residential areas with many parks, large gardens and small woods with low levels of urbanisation and, consequently, reduced air pollution, possibly functioning as a refuge from urban stressors. In contrast, squirrels captured in the peripheral urban area, located near a highway exit and adjacent to a six-lane arterial road, exhibited significantly higher levels of genomic damage. Similar results were reported for birds in forests fragments in central Brazil, where animals living in forests near urban areas presented higher micronuclei frequency (measured in erythrocytes) and a positive correlation was found between micronuclei occurrence and vehicle traffic intensity (Baesse et al., 2019).

Unfortunately, it was not possible to measure the concentration of chemical compounds in the soil of the agricultural areas included in the study directly, nor was it possible to assess airborne pesticide levels or determine the levels of other genotoxic pollutants (e.g. pesticides, polycyclic aromatic hydrocarbons and trace metals) in squirrels. However, these areas are known to be intensively used for cereal crop cultivation, where pesticides and other agrochemicals are widely applied. A study conducted in Mexico on the mammal species *Pteronotus mexicanus* linked exposure to agriculture with increased genotoxic effects in wildlife, thus supporting our rationale (Sandoval-Herrera et al., 2021). Indeed, populations of frugivorous and insectivorous bat species inhabiting human-altered environments showed high frequencies of micronuclei, and the genotoxic risk was likely influenced by diet and the type of disturbance (urban or croplands) (Benvindo-Souza et al., 2019). Moreover, a comparative study on fish, reptiles, amphibians and birds in Kazakhstan revealed multiple associations between different types of anthropogenic pollution sources. In particular, widest range of nuclear abnormalities (e.g. micronuclei frequency and cytological abnormalities) was associated with petrochemical and pesticide contamination (Cherednichenko et al., 2024). A survey was conducted among farmers who cultivate the fields around our study areas and with the addition of information obtained from agricultural land use in the Piedmont Region in 2021, 2022 and 2023, it was possible to determine the main types of cultivation occurring near the study areas: 1) Agri1 – maize, wheat, alfalfa (minor), barley (minor), soya (minor); 2) Agri2 – maize, wheat and asparagus. The list of agrochemicals applied in agricultural fields adjacent to the study areas is extensive (see Table S5). For many of these compounds, genotoxic effects have been documented in humans and/or experimental animals. Moreover, most studies typically assess the effects of individual active substances in isolation, whereas the potential synergistic or cumulative effects arising from simultaneous exposure to multiple compounds are rarely evaluated, despite their likely toxicological relevance for wildlife. Notwithstanding the limit imposed by the lack of assessment of pesticides levels in the environment and in squirrels, it is likely that grey squirrels consuming crops prior to harvest may be exposed to pesticide residues present on vegetative tissues. Common arable crops such as maize, wheat, alfalfa, barley, soya, and asparagus are frequently treated with herbicides, insecticides, and fungicides, many of which have demonstrated genotoxic potential (Hughes et al., 2011; Finger et al., 2019; Vasileiadis et al., 2016; Megersa, 2015). Evidence from mutagenicity and genotoxicity assays indicates that pesticides can induce DNA strand breaks, chromosomal aberrations, and oxidative DNA damage in mammalian systems (Boeira et al., 2001; Bolognesi, 2003; Das et al., 2007; Hartwig, 1994; Kirkland et al., 2015; Quinn-Hosey et al., 2012). Importantly, exposure to pesticide mixtures can result in genotoxic effects at lower concentrations than for single compounds, due to additive or synergistic interactions (Graillot et al., 2012; Kopp et al., 2018; Cedergreen, 2014; Hernández et al., 2017). This suggests that small mammals feeding on multiple pesticide-treated crops may experience cumulative genotoxic stress, which can be further exacerbated in cases of multiple sources of exposition, such as the case of study area Agri 1 where the water from a nearby river (Chisola) was used to irrigate cultivated fields. The parameters measured in the water (year 2021, Table S6) showed that several pesticides were present, as well as polycyclic aromatic hydrocarbons and metals, but in most cases at negligible concentrations. Conversely, in Agri 2 study area the water used to irrigate cultivated fields is mainly rainwater collected in an artificial lake. Therefore, a possible cumulative effect of different sources of exposition can be excluded. Although species-specific metabolism and actual residue levels influence the risk, chronic or repeated dietary exposure to pesticide residues can plausibly lead to genomic instability. Integrating analysis on squirrel tissues with biomarkers assays would allow a more precise evaluation of genotoxic risk in the environment.

To complement our assessment, we analysed air pollution data. Air pollutants, including $PM_{2.5}$, PM_{10}, NO_2, and O_3, can induce micronucleus (MNi) formation through both clastogenic and aneugenic pathways, primarily via oxidative stress, inflammation, and disruption of genome stability. Inhaled particulate matter generates reactive oxygen species directly and through redox-active constituents, leading to oxidative DNA lesions and strand breaks. If these lesions persist or are misrepaired before mitosis, acentric chromosome fragments may be excluded from daughter nuclei, resulting in clastogenic MNi formation (Fenech et al., 2011; Møller et al., 2014; Roginskaya and Razskazovskiy, 2023). In parallel, particulate pollutants such as PM_{10} can impair mitotic spindle function and spindle assembly checkpoint signaling, increasing chromosome mis-segregation and whole-chromosome loss, thereby promoting aneugenic MNi formation (Santibáñez-Andrade et al., 2022;

Vilas-Boas et al., 2024). Interestingly, levels of MNi and NBUDs in grey squirrels remained stable or showed a slight increase under conditions of low air pollution but decreased as air pollution levels rose. This non-linear pattern suggests a complex relationship between pollutant exposure and genomic biomarkers. Non-linear dose–response patterns, including hormetic-like responses, are well documented in toxicology and stress biology (Bondy, 2023). In ecological systems, biomarker responses often reflect a balance between damage induction and activation of compensatory mechanisms (e.g., antioxidant and DNA repair pathways), as well as possible selective survival of more resistant individuals. Therefore, a strictly linear increase in MNi/NBUDs with increasing pollution may not necessarily be expected (for a review see Guérard et al., 2015). Consistent with this framework, the observation of a non-linear pattern, characterised by an increase or stability in MNi/NBUDs at low pollution levels followed by a reduction at higher levels, can be interpreted as an "adaptive response" (Sutou et al., 2024). At low-dose, pollutant exposure may be insufficient to induce extensive DNA damage but sufficient to activate protective mechanisms (e.g., DNA repair, antioxidant defenses, and cellular turnover), resulting in a slight increase or stability in MNi and NBUDs. At higher doses, exposure may exceed tolerance thresholds, overwhelming defense systems and potentially suppressing micronuclei formation as damaged cells undergo necrosis/apoptosis prior to biomarker expression (Chapman et al., 2017; Sutou et al., 2024). This mechanism may account for both the non-linear curve observed and the lack of influence of individual variables (sex, age, body condition), which may become negligible compared to the intensity of pollutant exposure. Moreover, biomarker choice can critically influence the detected pattern. Field studies on free-living rodents have shown that responses are often both endpoint-specific and non-linear. In *Ctenomys minutus*, for example, DNA strand breaks (comet assay) increased under vehicular emissions, whereas MNi did not, reflecting different stages of damage detection by the two biomarkers (Heuser et al., 2002). Conversely, a study on laboratory mice showed a significant association between MNi frequency and air pollution levels (CO, NO_2, PM_{10}) (Soares et al., 2003). Hence, measuring the actual dose of contaminants in squirrels or assessing adaptive mechanisms, such as increased antioxidant enzyme concentrations and the upregulation of genomic damage repair systems, would contribute to clarifying the mechanisms involved.

Urban and agricultural areas impose different sources of anthropogenic disturbances on wildlife and, consequently, diverse environmental stressors. For instance, pollutants and pesticides, which are widely present in cities and cropfields, are known genotoxic agents that induce genomic damage/instability in animals (Cao et al., 2022; Rasgele, 2025), also trough the mediatory role of stress hormones (GCs). In the present study, however there was no effect of FGMs on MNi associated with different types of anthropogenic stressors (urban or agricultural areas). However, in the pairwise comparisons we found a weak difference of the relationship of FGMs with NBUDs between study area urban peripheral (UrbanP) and agricultural (Agri2).

The mechanistic relationship between genomic damage (MNi and NBUDs) and GCs remains complex. Glucocorticoids, through the action of their receptors, regulate inflammatory responses and cell survival, but prolonged elevation of GCs might compromise cellular integrity leading to high occurrence of nuclear abnormalities (Aprile-Garcia et al., 2014; Cruz-Topete and Cidlowski, 2015; Liberman et al., 2018). Several studies showed that pesticides and air pollutants can act as endocrine disruptors in wildlife (Köhler and Triebskorn, 2013) and are known for their impact on DNA and chromosomes (Baesse et al., 2019; Guilherme et al., 2014). Moreover, other factors such as oxidative stress mediate this relationship, possibly determining the observed lack of significance. Indeed, exposure to air particulate matter has been identified causing increase in reactive oxygen species (ROS) and inflammation, both contributing to produce DNA oxidation damage (Møller et al., 2014). For example, in fish, exposure to a common herbicide used worldwide produced an increase in ROS, lipid peroxidation, DNA damage and apoptosis (Martins et al., 2021). Furthermore, a study on laboratory rats showed that antioxidant defenses substantially decline following administration of glucocorticoids (corticosterone), reinforcing the role of GCs in induction of oxidative processes (Zafir and Banu, 2009), and thereby amplifying ROS exposure beyond that produced directly by pollutants. Taken together these findings highlight how our prediction of increase in genomic damage due to the synergic effect of GCs and different sources of anthropogenic disturbance (urban areas and cropfields) might be more complex and further investigations are needed to disentangle specific pathways.

5. Conclusions

Urban planning policies play a crucial role in shaping traffic distribution and air quality within cities. In an effort to reduce pollution, many initiatives have introduced regulations aimed at limiting vehicle access in city centres. These areas are typically characterised by high population density and work-related activity, and historically associated with elevated pollution levels (Lin et al., 2024). However, such measures can lead to the displacement of traffic congestion toward peripheral zones, increasing environmental burdens in these areas (Lampo et al., 2021).

While well-designed urban environments that incorporate accessible green spaces and limit traffic congestion are associated with improved public health outcomes (Iravani and Rao, 2020), urban policy efforts often focus disproportionately on central districts. As a result, peripheral neighbourhoods remain congested and continue to experience high levels of air pollution, with limited mitigation strategies in place. This uneven distribution of environmental quality highlights the need for more inclusive and spatially balanced urban planning approaches.

Policies efforts to mitigate the impact of anthropogenic disturbance must also consider alternative options regarding the usage of less genotoxic pesticides in agricultural practices. Indeed, the Piedmont region is characterised by arable crops and orchards interspersed with hedgerow and other semi natural elements which provide habitats for terrestrial mammals that are consequently exposed to agricultural pesticides. This exposure is supported by the detection of agrochemicals in hive matrices of honeybees (*Apis mellifera*) foraging in contaminated landscapes (Bergero et al., 2021). The present study focuses on an invasive mammal species, but highlights the importance of considering genotoxic effects when implementing policies to conserve also native species.

CRediT authorship contribution statement

Francesca Santicchia: Writing – review & editing, Writing – original draft, Software, Methodology, Formal analysis, Data curation, Conceptualization. **Alfredo Santovito:** Writing – review & editing, Writing – original draft, Validation, Resources, Methodology, Investigation, Data curation, Conceptualization. **Lucas A. Wauters:** Writing – review & editing, Supervision, Project administration, Investigation, Conceptualization. **Claudia Tranquillo:** Writing – review & editing, Visualization. **Rupert Palme:** Writing – review & editing, Resources, Methodology, Investigation, Data curation. **Damiano Preatoni:** Writing – review & editing, Software, Resources, Methodology, Formal analysis, Data curation. **Sandro Bertolino:** Writing – review & editing, Writing – original draft, Supervision, Project administration, Methodology, Investigation, Conceptualization.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Glossary

glucocorticoids (GCs)
micronuclei (MNi)
nuclear buds (NBUDs)
faecal glucocorticoid metabolites (FGMs)
enzyme immunoassay (EIA)
generalised linear mixed-effects models (GLMM)

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envres.2026.124414.

Data availability

The research data are available at: https://doi.org/10.5281/zenodo.17803127.

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