Impact of exposure to urban air pollution on grey squirrel (Sciurus carolinensis) lung health

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Keywords: grey squirrel, *Sciurus carolinensis,* air pollution, lung health, methylation, urban wildlife

Abstract

The increased rate of global urbanisation has recently exacerbated the significant public health problem of traffic related air pollution. Despite the known significant impact on human health, little is known about the effects of air pollution on wildlife health. The lung is the primary target organ for the effects of exposure to air pollution, leading to lung inflammation, altering the lung epigenome, culminating in respiratory disease. In this study, we aimed to assess lung health and DNA methylation profiles in Eastern grey squirrel (*Sciurus carolinensis*) populations living across an urban-rural air pollution gradient. Squirrel lung health was assessed in four populations situated across the most polluted inner-city boroughs to the less polluted edges of Greater London. We also assessed lung DNA methylation across three London sites and a further two rural sites in Sussex and North Wales. Lung and tracheal diseases were present in 28% and 13% of the squirrels respectively. Specifically, focal inflammation (13%), focal macrophages with vacuolated cytoplasm (3%) and endogenous lipid pneumonia (3%). There was no significant difference in prevalence of lung, tracheal diseases, anthracosis (carbon presence) or lung DNA methylation levels between urban sites and urban and rural sites respectively or distance from an A-road. BALT (Bronchus-Associated Lymphoid Tissue) size was smallest, with highest carbon loading in inner-London sites compared to outer-London sites, but this difference was not significant. However, high pollution site individuals had higher numbers of alveolar macrophages. We present preliminary evidence that urban squirrels are exposed and respond to traffic-related air pollution, but further research is needed.

Key words respiratory disease, traffic pollution, outdoor air quality, wildlife, invasive species

Introduction

Poor, and deteriorating air quality due to traffic-related pollution is the biggest environmental risk to health (WHO, 2017). Despite vast research in humans, to date there is limited empirical evidence measuring or quantifying the impact of urban air pollution on wildlife health, at either an individual or population level (Isaksson, 2015). Urbanisation continues to expand globally, particularly in species-rich areas, exposing a larger range of species, including threatened species, to traffic pollution (Hayhow et al., 2019). In recent decades, even urban adapted species have shown steep declines in abundance (e.g., butterflies, honeybees *Apis mellifera*, house sparrow *Passer domesticus*, common starling *Sturnus vulgaris* and hedgehog *Erinaceus europaeus*), with traffic-related air pollution (TRAP) being a potential unexplored risk factor (Hayhow et al., 2019; Peach et al., 2018). Historically, industrial air pollution (e.g., SO_2, arsenic, lead, smog, fluoride, and black carbon) has been shown to cause severe reductions in wild animal populations and in some instances extirpate them completely (Newman & Schreiber, 1984). The current gap in our understanding of how wild populations are affected by and respond to TRAP toxicity hinders our ability to effectively monitor, manage and predict an emergent risk to the health of all organisms.

Urban air pollution is largely a consequence of TRAP that contains a cocktail of ~~toxins~~ pollutants - ozone (O_3), particulate matter ($PM_{2.5}$ and PM_{10} e.g., black carbon), metals, polyaromatic hydrocarbons (PAHs) and nitrogen oxides (NO_x), the smallest particles of which can penetrate deep into the lung (WHO, 2017). These are all carcinogenic substances, thought to increase DNA damage and compromise DNA repair mainly through increased inflammation and levels of oxidative stress (Isaksson, 2015; Møller et al., 2014). Conditions in humans associated with TRAP exposure include respiratory inflammation, reduction of lung capacity, asthma, lung cancer, respiratory infections and the exacerbation of existing cardiopulmonary issues (Royal College of Physicians, 2016).

The limited studies on the impact of TRAP on wildlife generally mirror those from human studies (Isaksson, 2010). Studies on lung response in free-living populations of animals show higher levels of inflammation in the tissues of feral dogs (*Canis lupus*) (Calderón-Garcidueñas et al., 2003), feral pigeons (*Columba livia*) (Sicolo et al., 2010) and the Brazilian rodent (*Ctenomys minutus*) (Heuser et al., 2002) residing in areas with higher TRAP levels. The lungs' particle deposition and clearance mechanisms are largely dependent on alveolar macrophages and mucociliary clearance (Noël et al., 2016). Alveolar macrophages phagocytose particles derived from TRAP and trigger the body's innate immune response, providing the first line of defence against noxious air pollution (Bai et al.,

2015). Activated macrophages release inflammatory mediators which attract other immune cells to the site, and these elevated numbers of macrophages provide an excellent indicator of immune-activation and inflammation due to TRAP (Kulkarni et al., 2006). A study by Steyn & Maina (2015) (Steyn & Maina, 2015) of wild populations of house sparrows (*Passer domesticus*), Cape glossy starlings (*Lamprotornis nitens*) and laughing doves (*Spilopelia senegalensis*) in South Africa found higher numbers of alveolar macrophages present in the lungs of urban birds exposed to high TRAP levels. As well as lung exposure and responses to air pollution, TRAP exposure can also potentially alter lung DNA methylation levels.

DNA methylation is a widely studied epigenetic process, which through dynamic addition and removal of methyl group to cytosines remodelling can alter cell function by modulating transcription. Controlled DNA methylation remodelling mediates important processes such as cellular differentiation, development and healthy ageing (Wilson et al., 2007) but disfunction is associated with disease (Hanson et al., 2011).

Exposure to TRAP has been linked to alterations in DNA methylation patterns, in particular hypomethylation which is the loss of the methyl group in the 5-methyl cytosine nucleotide (Rider & Carlsten, 2019). A natural part of ageing (Jung & Pfeifer, 2015), hypomethylation has been causally linked to genetic instability and tumorigenesis (Rider & Carlsten, 2019). Any alteration to methylation levels due to external stressors has the potential for long-term negative impacts on an organism. The combination of inflammation, oxidative stress and epigenetic changes such as to DNA methylation, work in tandem to produce the disease outcomes associated TRAP exposure (Traboulsi et al., 2017). However, the underlying mechanisms or how these changes in DNA methylation influence inflammation, lung health and disease occurrence is still not well understood in humans or in other wild animals (Baccarelli et al., 2012; Rider & Carlsten, 2019).

Although we have strong evidence available regarding the cytotoxic and genotoxic effects that TRAP has in humans, domestic and laboratory animals, to our knowledge there are currently no studies evaluating the impact that TRAP exposure may have on lung ~~development~~ health and DNA methylation and disease outcomes in wild mammals. To fill this important gap in our understanding we examined if TRAP could explain variation in prevalence of lung disease and global lung methylation levels in seven wild populations of the invasive American Eastern grey squirrel (*Sciurus carolinensis*) occurring across an urban-rural air pollution gradient in the UK. The grey squirrel is an ideal model system to test the impact of air pollution: it occurs across all London green spaces, from the most polluted inner-city boroughs to the leafier edges of Greater London (Sheridan et al., 2019). They are also considered a pest and are systematically culled across all these sites because they damage property and the very trees that play such an important role in reducing air pollution in London (Merrick et al., 2016). Squirrels are exposed to realistic, complex levels of ambient air

pollution, the effects of which can be assessed histologically, something that human correlative studies and lab-based animal experiments rarely achieve as they can neither replicate the ambient air pollution 'cocktail' nor mimic the chronic exposure experienced by people and wildlife. Grey squirrel populations therefore provide a unique opportunity to assess the impact of exposure to TRAP on lung health.

Specifically, we ~~tested~~ assessed lung health by testing variation in 1) the presence or absence of black carbon in airway macrophages (anthracosis) and bronchus-associated lymphoid tissue (BALT); 2) the number of alveolar macrophages, BALT size and BALT to lung size ratio, as well as, global lung methylation levels and whether 3) the presence or absence of tracheal and lung diseases, were explained by average levels of NO_2 at each site, distance from each cull site to a major road (used as a proxy for TRAP exposure) as well as an individual's ~~squirrel's age, weight and~~ sex.

~~Material and~~ Methods

~~Species and study areas~~Study species

The Eastern grey squirrel is an invasive rodent first introduced to the British Isles in the late 1800s. Multiple introductions, by private landowners, as an ornamental species, led to the establishment and expansion of grey squirrel populations. Grey squirrel's now range across most of England, Wales, and eastern Ireland (Signorile et al. 2016).

~~In the UK, t~~The presence of grey squirrels negatively affects native ecosystems, as they outcompete and spread disease to the~~the indigenous~~native red squirrel (*Sciurus vulgaris*) and inflict significant damage to woodlands and parks via bark stripping (Bertolino & Genovesi, 2003; Tompkins et al., 2002). Current population size estimates in the UK range from 2-3 million individuals distributed along the rural-urban gradient (Merrick et al., 2016), and numbers are managed with systemic culling across the country to reduce forestry damage and prevent the local extinction of red squirrels (Mill et al., 2020).

Sampling

Sampling was done in two phases. Lung samples acquired in the first phase in Spring (February-May) of 2015 and 2017 were used for the global methylation analysis. These were from three urban boroughs across London (Camden = 2; Greenwich = 4 and Richmond = 15) and two rural sites in Surrey (Alice Holt = 12) and North Wales (Penrhyn Castle = 12). In the second phase, samples were acquired for histopathology in the Spring of 2019 and 2020 and Summer (June-July) of 2019, f~~. F~~rom four urban boroughs across London (Westminster = 13; Greenwich = 20; Haringey = 19; Richmond = 9) (Table S1). Each site was selected based on whether a culling scheme was in place.

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Pollution metrics

A total of 106 individuals were sampled in the Spring and Summers between 2015 and 2020, in five London Boroughs (Camden, Greenwich, Haringey, Richmond-Upon-Thames and Westminster) and two rural sites (Alice Holt in Sussex, Southern England and Penrhyn Castle in Gwynedd, North Wales) (Figure 1).

~~A total of 1067 individuals were sampled in the Spring and Summers between 2015 and 2020, in five London Boroughs (Camden, Greenwich, Haringey, Richmond-Upon-Thames and Westminster) and two rural sites (Alice Holt in Sussex, Southern England and Penrhyn Castle in Gwynedd, North Wales) (Figure 1 and Figure 2). Each site was selected based on whether a culling scheme was in place~~ Each site was selected based on t~~and t~~he annual average NO_2 level, acquired from DEFRA's Automatic Urban and Rural Network (AURN), and the King's College London Air Quality Network (LAQN) (Figure 1~~ and Figure 2~~). ~~Average NO_2 levels and distance to the nearest A-road were used as a proxy for levels of traffic-related air pollution in each site. Distance from the sampling site to the nearest A-road (i.e. major road) in metres was determined using Google Maps.~~Readings were accessed via online databases then an overall average was taken to cover the subject's exposure to NO_2 in the year prior to being culled. Rural levels were taken from the AURN database. Urban levels were acquired from the LAQN database, annual averages were taken from the readings produced by the nearest monitoring stations (daily NO_2 ug m^{-3}) to the site of specimen acquisition. Due to the sporadic nature of the monitoring stations, particularly in rural areas, it was not possible to get exact data for the locations of specimen collection. Instead, data was acquired from the closest monitoring station. NO_2 was used as a proxy for air pollution exposure as it is directly correlated to a large number of other vehicle emission pollutants and one of the few pollutants consistently monitored across monitoring stations (~~Supplementary~~ Table S1). Average NO_2 levels and distance to the nearest A-road (i.e., major roads intended to provide large-scale transport links within or between areas) were used as a proxy for levels of traffic-related air pollution in each site. Distance from the sampling site to the nearest A-road in metres was determined using the measuring tool in Google Maps.

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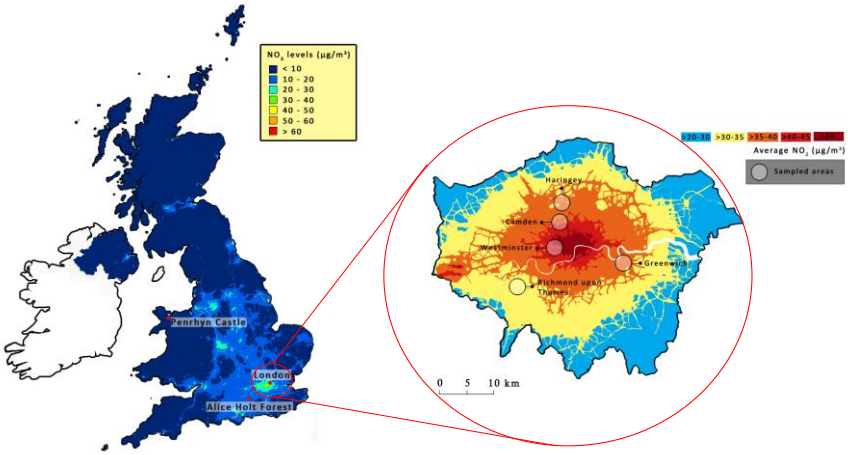

Figure 1. Map of the United Kingdom and Greater London with locations of the sample sites (in England with red dots, and London open circles) with a background showing the annual average concentration of NO_2. The data used in this map was extracted from the London Atmospheric Emissions Inventory (2016).

Post-mortem examination

Each grey squirrel was weighed (*g*) and sex was determined from morphology. Age was determined by examining the extent of epiphyseal fusion by radiograph (Dubock, 1979). The epiphyseal gap of the radius and ulna were measured (in millimetres) using ImageJ software (Schneider et al., 2012). Depending on the size of the gap, three different age categories were obtained: 1 (0-27 weeks of age), 2 (28-48 weeks) and 3 (49 weeks or older). Post-mortem examinations were carried out on 61 individuals. Examinations assessed sex, ~~abnormalities~~abnormalities, and the presence of gross macroscopic lesions in all major organs (Table S5). The lungs were removed and immersed in 10% neutral-buffered formalin and stored at room temperature. ~~For the global methylation assay, left lung lobe samples were taken from an additional 45 individuals. Lung tissue samples were~~and were ~~stored in 70% ethanol at -20ºC until processed.~~

Histopathology

Grey squirrels only possess one lobe on the left lung and four lobes on the right lung (Figures S1.1-1.3). Formalin-fixed lung tissue samples from 61 grey squirrels across four locations in London were embedded in paraffin wax, sectioned at 4 *µm* ~~slices~~ and stained routinely with haematoxylin and eosin. Sections from the middle part of the trachea; cranial and caudal area of the left lung; middle part of the cranial, middle, caudal and accessory right lung lobes of each squirrel were taken. Histopathology slides were digitally scanned and reviewed using the NDP.view 2 software (Hamamatsu.com, 2020). Slides were produced for each lung lobe, which included the main bronchi to assess the Bronchus-Associated Lymphoid Tissue (BALT). Lung diseases were identified by the presence and type of inflammatory cells, as well presence of lesions and their distribution (diffuse or local). Tracheal diseases were identified by attenuation ~~flattening~~ of the epithelium (erosion), presence of inflammatory cells and/or ulceration in the respiratory epithelium.

Slides were also screened for the presence of black carbon particles in the alveolar macrophages and BALT tissue (anthracosis). As well as the number of alveolar macrophages, size of the BALT tissue (if present), total lung size per slide and the BALT:lung area ratio was estimated using the NDP.view 2 "Freehand region" tool. BALT area and lung area were assessed to develop a BALT:lung ratio and determine the size of the BALT in relation to the lung size estimates. Macrophage counts were performed by randomly selecting an area of $8x10^{-7}$ m^2 (0.8 mm^2) per lung section, and the number of alveolar macrophages within this area counted at 40x magnification to obtain numbers per lung unit.

Global DNA methylation assay

For the global methylation assay, left lung lobe samples were taken from 45 individuals and were stored in 70% ethanol at -20ºC until processed. DNA was extracted from 20 *mg* of tissue from the upper left lung lobe, from each individual, using the Qiagen DNeasy Blood & Tissue kit following the manufacturer's instructions and stored at 20ºC. Concentration of DNA samples was quantified using a Qubit 2.0 Fluorometer and 100ng of each sample used to undertake the assay. Obtaining the concentration of DNA in each sample informed specimen selection for the assay, as well allowing for the calculation of the DNA to AE buffer ratio that was needed in each well. Global DNA methylation was quantified in each lung sample, using the Epigentek MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Epigentek, USA). A 96-well assay was carried out, with the samples randomised across the plate to minimise bias. 10% of samples were repeated to act as controls. 100ng of DNA was used per well, and the assay was carried out as per the manufacturer's

instructions. The resultant colour change, which indicates the relative abundance of methylated cytosine, was quantified using a BioTek absorbance plate reader, with the colour intensity measured at 450 nm. Raw values were converted into percentage of 5mC in total DNA using a standard curve of known concentrations of methylated DNA. The data then had to be converted to a 5-mC/(5-mC+C) format, where the 5-mC% was divided by a known cytosine content. The cytosine content of human DNA, at 21% was used a proxy.

~~Global methylation levels (5C-methylation and 5C-hydroxymethylation) of every sample were measured using an ELISA-based colorimetric method in a 96-well plate (Epigentek, USA). The colour change, which is relative to abundance of methylated cytosine, was quantified using a plate reader (BioTek absorbance plate reader). Colour intensity was measured at 450nm. Raw values were converted using a standard curve of methylated DNA of known concentrations.~~

~~***Statistical*** ***Data*** ***analyses***

Does lung health vary between populations living at a gradient of urban air pollution?

Lung health

Generalized linear models were used to examine differences in lung health between urban populations of grey squirrels living ~~at~~across a gradient of air pollution. Based on histopathology data, the presence of absence of a) black carbon particles within BALT tissue, b) alveolar macrophages, c) tracheal ~~lung~~ disease and d) lung disease were all tested as Binomial response variables. Models contained distance from an A-road, NO_2 levels and sex as explanatory variables. Interactions between sex and site were also tested, to assess whether sampling differences and differences in lung size between sexes/populations had an impact. Final models were selected using AIC values using the MuMIn package in R version 1.4.1106 (R Core Team, 2021).

~~We conducted all statistical analyses in R version 4.1.0 (R Core Team, 2021). We used generalized linear models with a Binomial distribution to examine whether presence or absence of black carbon particles within the BALT tissue, alveolar macrophages, as well as presence of tracheal and lung disease were associated with individual population location, distance from an A-road, sex, and age.~~

To assess differences in the number of airway macrophages per lung area (0.8 mm^2), the BALT and lung area and BALT to lung area ratio, we used ~~generalized~~ linear model~~s~~ ~~with Poisson distribution~~ with individual population ~~location~~, sex, and ~~age~~ NO_2 as explanatory variables. The severity of black carbon particle~~s~~ deposition within BALT tissue was tested using an ordinal logistic regression using the MASS package in R version 1.4.1106 (R Core Team, 2021). Models contained

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NO_2 levels and sex as explanatory variables. Weight was not included in the models as it was highly correlated with levels of NO_2 with larger individuals found in areas of lower NO_2. Interactions between sex and levels of NO_2 pollution were also tested.

Lung methylation

Given the small number of global methylation samples in some urban sites (see Table S2), the values for either urban or rural sites were pooled together. ~~DNA methylation data was tested for normality using visual analysis with histograms and boxplots and Shapiro-Wilk test. Skewness and kurtosis were tested using D'Agostino test for skewness and Bonett-Seier test of Geary's kurtosis, respectively.~~ DNA methylation data had a righthand-skew distribution, we therefore used~~did not follow a normal distribution and had right-handed skewness.~~ ~~Following test of normality, DNA methylation data was found not to be normally distributed, with a tendency for a right-handed skew. We then used~~ Generalized Linear Models with Gaussian distribution to compare if there was difference in mean global DNA~~We therefore used a non-parametric Wilcoxon-Mann-Whitney test to compare two independent samples to compare if there was difference in mean global DNA~~ methylation levels between urban and rural groups, males and females and~~, distance to~~ A-road and~~ and~~ individuals of different age. We also tested if interactions between urban and rural sites and sex~~ the whether the distance from an A-road and interaction between urban and rural sites and sex~~, and rural and urban sites and age predicted DNA methylation levels~~ using generalized linear models with a Gaussian distribution.~~ Final models were selected using AIC values using the MuMIn package in R version 1.4.1106 (R Core Team, 2021).

~~We used the Akaike Information Criterion (AIC) to choose the most parsimonious model. .~~

Results

Histopathology

A total of 61 squirrels (27 females and 34 males) were ~~screened~~ examined from four different locations across London between 2019-2020 (~~Table 1, S1~~~~Supplementary~~ Table S2). Lung and tracheal lesions were present in 28% (17/61 animals) and 13% (8/61 animals) of the squirrels, respectively. Specifically, focal inflammation (13%), focal macrophages with vacuolated cytoplasm (3%) and endogenous lipid pneumonia (3%) (Table S5). Cases of lung and tracheal disease tended to be higher in Westminster (~~Supplementary~~ Table S2). Anthracosis (black carbon, Figure 2A-C) was present in 16% of the BALT samples and 14% of the total alveolar macrophages screened. However, anthracosis quantification in each alveolar macrophage was not assessed as not enough

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cells with black carbon were found. Black carbon presence in the BALT tended to occur more in individuals from Westminster ~~(Figure 2 and 3)~~ and black carbon in alveolar macrophages (AM) was more commonly found in individuals from Haringey (~~Supplementary~~ Table S2). The effects of air pollution on lung health were formally tested using a series of Binomial models. Distance from an A-road, weight and NO_2 were all highly correlated. We therefore proceeded with the metric most closely associated with air pollution indices (NO_2 only). All the top models (based on ΔAIC < 2) contained NO_2 as an explanatory variable (Table 1). However, none were strongly supported. In the models with the lowest AIC values, we found a significant trend towards the effect of annual levels of NO_2 prior to the cull date across each site on the number of alveolar macrophages and BALT area (Figure 2A-C) within the lung (Table 2). With individuals living in more polluted sites having a higher number of alveolar macrophages and smaller BALT area (Figure 3). NO_2 levels differences between sites did not seem to have an impact on the levels of tracheal or lung disease, lung area, BALT:Lung ratio or the amount of carbon particles found in the alveolar macrophages or within the BALT (Table 2).~~As expected, juveniles had a significantly smaller lung size than adults (Lung area: *slope* = 0.68; *SE* = 0.07; *Z-value* =9.04; *p-value* = 0.001), and although sexual size dimorphism is not prominent in this species, males had significantly larger lungs than females (Males: *slope* = 0.11; *SE* = 0.04; *Z-value* = 2.55; *p-value* 0.05). However, there was no significant difference in lung size between individuals from different locations (Westminster: *slope* = - 0.02; *SE* = 0.05; *Z-value* = - 0.53; *p-value* = 0.98) or sampled at different distances from an A-road (*slope* = - 0.0001; *SE* = 0.0001; *Z-value* = - 1.89; *p-value* = 0.23). BALT size was smallest in Westminster and largest in Haringey squirrel populations (Table 1), but did not vary significantly with location (Westminster: *slope* = - 0.79; *SE* = 1.27; *Z-value* = - 0.61; *p-value* = 0.53; Figure 3), or with age (*slope* = 0.60; *SE* = 1.48; *Z-value* = 0.40; *p-value* = 0.68) or distance to an A-road (*slope* = -0.0003; *SE* = 0.0002; *Z-value* = - 0.16; *p-value* = 0.87). BALT to lung size ratio was also not predicted by location, distance to A-road, sex or age.~~

~~Squirrels from Westminster, the site with highest levels of traffic air pollution, showed significantly higher number of alveolar macrophages per lung section (Westminster: *slope* = 0.34, *SE* = 0.14, *Z-value* = 2.40, *p-value* = 0.01) than those in the other three less polluted populations (Richmond: *slope* = 0.31, *SE* = 0.24, *Z-value* = 1.25, *p-value* = 0.21). Lung and tracheal lesions were present in 28% (17/61 animals) and 13% (8/61 animals) of the squirrels respectively. Specifically, focal inflammation (13%), focal macrophages with vacuolated cytoplasm (3%) and endogenous lipid pneumonia (3%). Cases of lung and tracheal disease tended to be higher in Westminster (Table 1). However, there was no significant difference in their presence of either disease between sites (Lung disease: *slope* = - 2.38; *SE* = 0.00; *Z-value* = - 0.18; *p-value* = 0.85) (Tracheal disease: *slope* = 2.07; *SE* = 1.79; *Z-value* = 1.15; *p-value* = 0.24), distance from A-road (Lung disease presence:~~

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~~*slope* = 0.00; *SE* = 0.00; *Z-value* = - 1.04; *p-value* = 0.29) (Tracheal disease: *slope* = 0.009; *SE* = 0.005; *Z-value* = 1.71; *p-value* = 0.08), age or sex of the squirrels. Anthracosis (black carbon, figure 2) was present in 16% of the BALT samples and 14% of the total alveolar macrophages screened. However, anthracosis quantification in each alveolar macrophage was not assessed as not enough cells with black carbon were found. Black carbon presence in the BALT tended to occur more in individuals from Westminster and black carbon in alveolar macrophages (AM) was more commonly found in individuals from Haringey (Table 1). However, black carbon presence in BALT (BALT carbon: *slope* = - 0.85; *SE* = 1.23; *t-value* = - 0.69; *p-value* = 0.48) and alveolar macrophages (AM with carbon: *slope* = - 2.58; *SE* 1.47; *Z-value* = - 1.75; *p-value* = 0.079) did not vary significantly between sites, with distance from an A-road (BALT carbon: *slope* = - 0.006; *SE* = 0.003; *t-value* = - 1.74; *p-value* = 0.08; AM carbon: *slope*: 0.00; *SE* = 0.00; *Z-value* = 0.13; *p-value* = 0.89) or with age or sex of the squirrels.~~

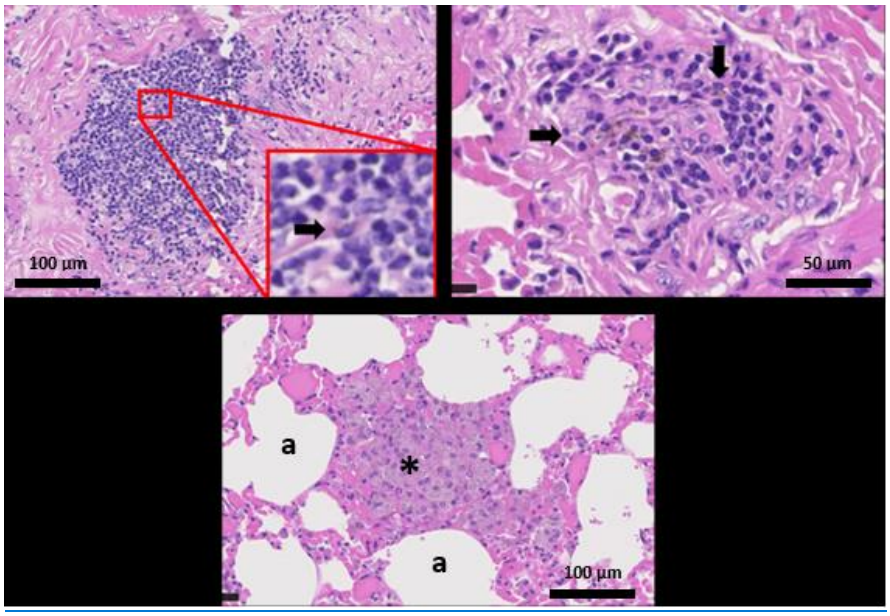

Figure 2A-C. A. Localised intracytoplasmic carbon particles (black arrow) found in lymphocytic cells contained in lung BALT tissue. B. Multiple foci of intracytoplasmic carbon particles (black arrows) found in BALT lymphoid tissue. C. Macrophages with foamy cytoplasm containing carbon particles (*), found in the lung parenchyma. a: alveoli. ~~Figure 2A-C. A. Localised intracytoplasmic carbon particles (black arrow) found in lymphocytic cells contained in lung BALT tissue. B. Multiple foci of intracytoplasmic carbon particles (black arrows) found in BALT lymphoid tissue. C. Macrophages with foamy cytoplasms full of carbon particles (*), found in the lung parenchyma. a: alveoli.~~

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Table 1 Models used to test the effects of air pollution on lung health. Models selected on lowest delta AIC (> 2). NO_2 experienced per site in the year before the cull and individual sex were used as fixed effects as well as the interaction between the two.

Tracheal disease			***Lung disease***		
Model formula	**AIC**	**ΔAIC**	**Model formula**	**AIC**	**ΔAIC**
Tracheal disease~NO2	52.73	0.0	Lung disease~Sex	59.03	0.0
Tracheal disease~Sex	53.45	0.7	Lung disease~NO2	60.19	1.2
Tracheal disease~Sex+NO2	54.73	2.0	Lung disease~Sex*NO2	60.34	1.3
			Lung~Sex+NO2	60.9	1.9
Alveolar macrophage (AM) carbon			***Alveolar macrophage***		
Model formula	AIC	ΔAIC	Model formula	AIC	ΔAIC
AM_carbon~NO2	40.52	0.0	Alveolar macrophages~Sex*NO2	291.69	0.0
AM_carbon~Sex+NO2	41.83	1.3	Alveolar macrophages~Sex	291.87	0.2
AM_carbon~Sex	42.19	1.7	Alveolar macrophages~NO2	292.64	0.9
			Alveolar macrophages~Sex+NO2	293.68	2.0
Lung area			***Balt:Lung ratio***		
Model formula	AIC	ΔAIC	Model formula	AIC	ΔAIC
Lung area~Sex	460.04	0.0	BALT:Lung ratio~Sex*NO2	-449.68	0.0
Lung area~Sex*NO2	460.84	0.8	BALT:Lung ratio~Sex+NO2	-448.8	0.9
Lung area~Sex+NO2	461.49	1.4	BALT:Lung ratio~Sex	-448.3	1.4
			BALT:Lung ratio~NO2	-448.08	1.6
BALT area			***BALT carbon***		
Model formula	AIC	ΔAIC	Model formula	AIC	ΔAIC
BALT area~NO2	-25.2	0.0	BALT carbon~Sex	78.97	0.0
BALT area~Sex+NO2	-23.25	2.0	BALT carbon~NO2	79.94	1.0
			BALT carbon~Sex+NO2	80.96	2.0

Table 2 The results of eight separate models testing for the effect of NO2 exposure and sex on lung health. Significant effects are shown in bold.

	Alveolar macrophages			**Lung area**			**BALT area**		
Factor	Estimate	SE	P value	Estimate	SE	P value	Estimate	SE	P value
NO2	0.221	0.1	**0.037**	-	-	-	-0.003	0.001	**0.036**
Sex	7.671	4.79	0.118	22.77	13.35	0.096	-	-	-
Sex:NO2	-0.247	0.13	0.059	-	-	-	-	-	-

	Tracheal disease			**Lung disease**			**AM Carbon**		
Factor	Estimate	SE	P value	Estimate	SE	P value	Estimate	SE	P value
NO2	0.018	0.02	0.382	0.023	0.02	0.233	-0.04	0.037	0.217
Sex	-	-	-	-	-	-	-	-	-
Sex:NO2	-	-	-	-	-	-	-	-	-

	BALT:Lung ratio			**BALT carbon**		
Factor	Estimate	SE	P value	Estimate	SE	P value
NO2	9.01E-06	2.25E-05	0.691	-	-	-
Sex	8.77E-04	1.05E-03	0.409	-0.57	0.588	0.326
Sex:NO2	-4.94E-05	2.79E-05	0.107	-	-	-

Table 1 Histopathology summary results, for 61 grey squirrels across four London sites between 2019-2020. Additional variables are sample size (N), pollution levels (NO_2), distance to A-road, number of male (M) and female (F) squirrels and percentage of adults per sample site.

Site	Year	N	Range of NO_2 µL/m^2	Distance to A-road (m) ± SE	M	F	% adults	Weight (g) ± SE	AM	Tracheal disease	Lung disease	Carbon in BALT	Carbon in AM	BALT size (mm^2) ± SE	BALT:lung size ratio
Westminster	2019	13	40-45	462 ± 48	9	4	72	574 ± 18	15.23	4/12	5/13	5/10	1/10	0.13 ± 0.04	0.002
Greenwich	2020	20	35-40	200 ± 0	11	9	NA	533 ± 17	12.4	1/18	7/20	0/15	1/20	0.23 ± 0.03	0.003
Haringey	2019	19	35-40	246 ± 41	7	12	87	589 ± 17	12.24	1/14	4/19	3/19	5/19	0.27 ± 0.05	0.003
Richmond	2019	9	30-35	976 ± 92	7	2	100	552 ± 17	12.74	2/9	1/8	1/7	1/9	0.24 ± 0.07	0.002

Number of individual squirrels sampled (N) and range of annual NO_2 pollution (in µL/m^3) in each site. The average distance in meters from collection site to the closest A-road (Distance to A-road) with standard error (SE). Number of sampled males (M) and females (F), percentage of individuals older than 49 weeks (% adults) and average body weight in grams (Weight (g)) of sampled individuals per site. Number of alveolar macrophages per 0.8mm2 lung section (AM). Cases of tracheal and lung disease, black carbon presence in BALT and alveolar macrophages in total number of individuals assessed (# positive/total). BALT size in mm^2 with 95% confidence interval (CI) and BALT size to total lung size ratio.

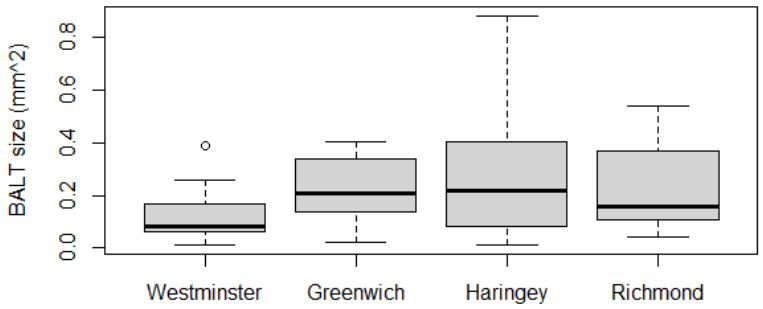

Figure 3. Boxplot showing 25th and 75th percentile, with whiskers denoting the maximum and minimum value of the median grey Squirrel lung BALT size (in mm^2) for each London borough sampled. Boroughs have been ordered from inner London to outer London (from most to least polluted sites).

Global DNA methylation

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A total of 45 squirrels (17 Females and 28 Males) were sampled in two rural sites and three urban sites ~~(Table 2)~~. Penrhyn Castle was the most rural site and had the highest global DNA methylation levels while urban sites in Camden and Richmond had the lowest (~~Supplementary~~ Table S3~~2; Figure 4 a, b~~). The effects of air pollution on lung global methylation were formally tested using a series of GLM~~M~~s with NO_2, distance from and A-road, urban/rural site, age, sex and weight as fixed effects. NO2, distance from an A-road and weight were corelated and therefore tested separately (Table 3). The top models (based on ΔAIC < 2) contained NO_2, urban/rural site, sex, and weight as explanatory variables (Table 3). However, none of the fixed effect variables were strongly supported (Supplementary Table 4). Lung global DNA methylation does not vary consistently with air pollution metrics.~~However, pooled rural and urban locations showed no significant difference in mean lung global DNA methylation levels (Wilcoxon-Mann-Whitney test: w = 238; p-value = 0.94); between males and females (Wilcoxon-Mann-Whitney test: *w* = 244; *p-value* = 0.73) or between different age categories (Wilcoxon-Mann-Whitney test: *w* = 212; *p-value* = 0.66). DNA methylation levels were not predicted by distance to an A-road (DNAm: *slope* = - 0.061; *SE* = 0.49; *t-value* = -1.25; *p-value* = 0.90) and there was a not significant trend towards higher methylation levels in rural females compared to urban females, that was not reflected in males (Interaction between location and sex: *slope*: 2.54; *SE* = 1.43; *t-value* = 1.76; *p-value* = 0.08; Figure 4a). Global DNA Methylation levels also did not vary between young and old individuals in rural or urban sites (age by site interaction: *slope* = - 0.69; *SE* = 0.77; *t value* = - 0.90; *p value* = 0.37; Figure 4b).~~

Table 3 Models used to test the effects of air pollution on lung global methylation levels. Models selected on lowest delta AIC (> 2). Sex, weight, NO_2 experienced per site in the year before the cull and urban/rural populations were used as fixed effects as well as the interaction between the latter and sex.

Model formula	AIC	ΔAIC
DNA Methylation~Sex	195.21	0.00
DNA Methylation~Weight	195.25	0.04
DNA Methylation~NO2	195.28	0.07
DNA Methylation~Urban/rural population	195.42	0.21
DNA Methylation~Urban/rural population*Sex	195.75	0.54
DNA Methylation~Sex+Weight	196.97	1.76

~~**Table 2** Lung global DNA methylation results summary table for 45 grey squirrels sampled between 2015-2017 in two rural and three urban sites, including NO_2 pollution levels, number of male and female squirrels and squirrel weight (g).~~

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Site	Year	N	Range of NO^2 µl/m^3	Distance to A-road (m) ± SE	U	M	F	% adults	Weight (g) ±SE	Average mDNA ±SE
Penrhyn Castle	2015	12	>20	1020 ±0	R	6	6	60	540 ±20	3.8 ±1.1
Alice Holt	2017	12	>20	754 ±84	R	6	6	80	497 ±41	3.2 ±1.2
Greenwich	2017	4	35-40	190 ±31	U	4	0	50	462 ±57	3.2 ±0.6
Camden	2017	2	40-45	262 ±0	U	0	2	100	596 ±4	3.1 ±2.7
Richmond	2017	15	30-35	649 ±28	U	12	3	80	572 ±11	3.1 ±0.6

Number of individual squirrels sampled (N) and range of annual NO_2 pollution (in µL/m^3) in each site. The average distance in meters from trap-site to the closest A-road (Distance to A-road) with standard error (SE) and whether the site is in an urban (U) or rural location (R). Number of sampled males (M) and females (F), percentage of individuals older than 49 weeks (% adults) and average body weight in gramsms (Weight (g)) with SE of sampled individuals per site. Average lung methylation level with SE per site.

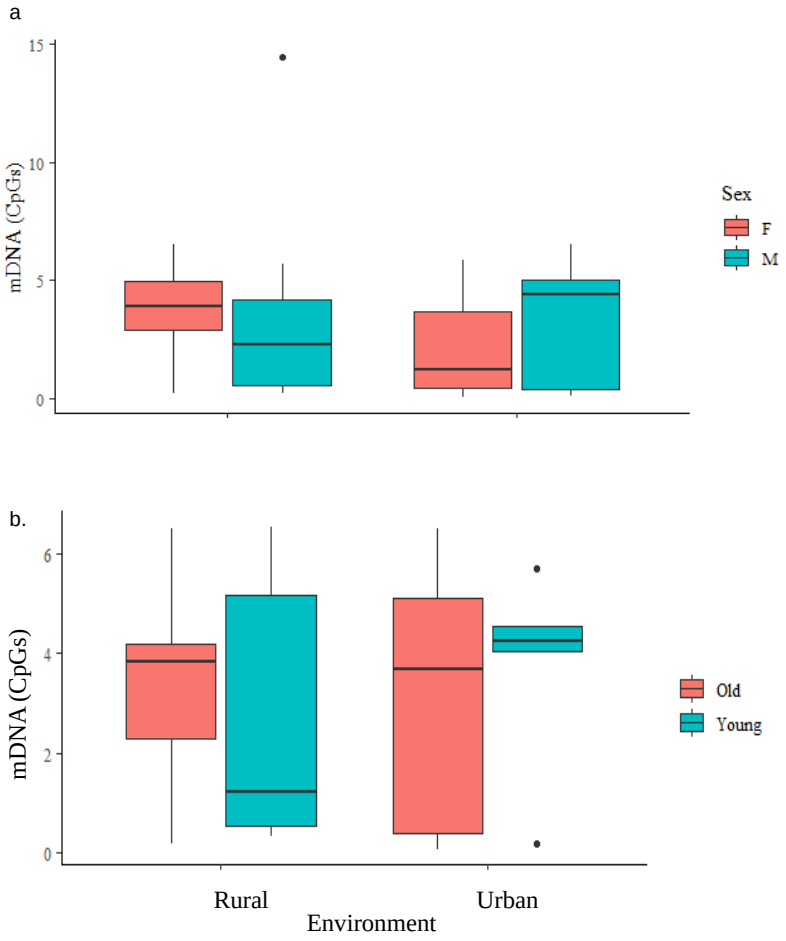

Figure 4 Boxplot showing median lung DNA methylation levels in (a) male and female individuals inhabiting urban and rural sites, with 25th and 75th percentiles and whiskers showing maximum and minimum values, and in (b) young and old individuals inhabiting urban and rural sites. Young individuals range from 0-27 weeks of age and old are older than 49 weeks of age.

Discussion

~~Overall, urban grey squirrel populations exposed to traffic-related air pollution (TRAP) have a high prevalence of lung and tracheal diseases. However, we~~We found no evidence for a significant difference in lung or tracheal disease prevalence between urban populations living across a

gradient of air pollution or evidence for significant differences between urban and rural populations of grey squirrels in levels of lung DNA methylation. However, populations with a higher exposure to TRAP from Westminster in central London had a significantly higher number of alveolar macrophages and a tendency for reduced BALT size with a higher number of black carbon particles than the populations exposed to lower TRAP levels in London. This indicates that grey squirrels are exposed to and respond to urban air -pollution, but we cannot definitively link it to disease prevalence without extending this study to measure prevalence of lung and tracheal diseases in rural populations with much lower air pollution exposure levels than those in urban settings.

Black carbon in BALT and alveolar macrophages is used as a standard metric of direct individual exposure by inhalation of TRAP in humans (Bai et al., 2015) and laboratory species (Decaesteker et al., 2021). In humans, black carbon in alveolar macrophages is usually assessed using bronchoalveolar lavage (BAL), as more invasive sampling is not possible (Bai et al., 2015). In this study, we found limited evidence of black carbon inhalation with 18% of squirrels showing black carbon particles in the BALT and 17% of squirrels showing black carbon particles in alveolar macrophages. However, black carbon loading of the alveolar macrophages was minimal. Black carbon particles tended to be found in a larger proportion of individuals from Westminster (50% of individuals had black carbon in the BALT) and Haringey populations (26% of individuals had black carbon in alveolar macrophages). Both populations are closer to major roads when compared to the Richmond population. However, the Greenwich population had very little evidence of black carbon in either the BALT or alveolar macrophages, despite also being close to high traffic areas. Our samples have very low levels of black carbon compared to those from human studies in London, UK (Brugha et al., 2014; Nwokoro et al., 2012) and tree sparrows (*Passer montanus*) in the Hebei province of China (Li et al., 2021). Potentially due to differences in sampling technique, with alveolar macrophages in BALT likely presenting higher black carbon loading than those fixed in histopathology tissue. Humans and their companion animals (such as pet dogs) may also experience higher exposure and accumulation levels as they are more closely associated with major roads and live longer (Calderón-Garcidueñas et al., 2001) than grey squirrels that have a level of buffer from inhabiting the tree canopies in green spaces of urban areas (Merrick et al., 2016).

Exposure to vehicle emissions induces an inflammatory response in the airways and lungs of humans, laboratory species and companion animals (Clarke et al., 1999; Hiraiwa & van Eeden, 2013; Reif, 2011). Characterised by neutrophil, lymphocyte, and mast cell influx into the airways as these form the first line of cellular defence of the mammalian lung (Kelly & Fussell, 2015). Here, we show a significantly higher number of alveolar macrophages in the lungs of squirrels living in more polluted areas of central London (Westminster), when compared to populations with lower TRAP

exposure. This indicates that squirrels in this area are responding to external airborne agents. In wild populations, exposure to urban air pollution has been shown to increase the number of circulating alveolar macrophages (Lorz & López, 1997; McArn et al., 1974; Steyn & Maina, 2015), also lower the number of lung lamellar bodies (Lorz & López, 1997) and have no effect on lung oxidative damage (Isaksson et al., 2009) in birds. Experimental exposure to TRAP also ~~suppressed~~ reduced T-cell mediated immune response in the skin of European starlings (*Sturnus vulgaris*, (North et al., 2017). Therefore, it is likely that exposure to TRAP induces a heightened alveolar macrophage response or a combination of TRAP exposure and disease susceptibility in the Westminster squirrel population.

Stress and inflammation associated with urban living (Isaksson, 2015) and exposure to TRAP elicits epigenetic changes, specifically DNA methylation in humans, laboratory, and wild animals (Ji & Khurana Hershey, 2012; Jiang et al., 2014; Romano et al., 2017) and linked to accelerated ageing (Ward-Caviness 2016). Generally, exposure to TRAP leads to hypomethylation in exposed tissues (Baccarelli et al., 2012; Ji & Khurana Hershey, 2012; Ding et al., 2016). Due to the ease of sampling, the bulk of previous studies in humans and mice models have focused on blood samples, with a negative association with TRAP exposure reported in both global DNA methylation, and that of repetitive DNA elements such as LINE1 and Alu (Ding et al., 2016). DNA methylation patterns tend to be cell specific (Rider & Carlsten 2019), and hence we tested global DNA methylation directly in lung tissue as more likely to be impacted directly by air born pollutants. However, we found no difference in lung global DNA methylation levels between urban and rural populations of grey squirrels. Potentially site-specific changes in methylation may have occurred and gone undetected due to the lack of specificity of the laboratory techniques used in this study. However, this lack of differentiation between sites does reflect findings from a study conducted on Wistar rats (*Rattus norvegicus*), who were subjected to variable degrees of traffic pollution. The rats exposed to the highest level of PM presented with demethylation in the iNOS promoter in blood, but no difference in lung tissue (Tarantini et al., 2009; Ding et al., 2016). Due to the respiratory effects associated with TRAP exposure, it is highly unlikely that DNA methylation is completely unaffected. However, changes in methylation patterns may occur in specific regions or genes rather than globally. Further study is required to fully understand to the gene-specific epigenetic consequences of TRAP exposure on the lungs.

Exposure to TRAP has been shown to directly affect tracheal epithelium, shorten airway cilia (Llacuna et al., 1996) and lead to the development of lung carcinomas (Reymão et al., 1997) in wild rodent populations. We did not find any difference in the prevalence of disease among London populations of grey squirrels. However, overall prevalence of tracheal (13% of individuals) and localised lung disease (28% of individuals) across these urban populations is high compared to

other studies of wild squirrels in less urbanised and rural areas in the UK (Blackett et al., 2018; Shuttleworth et al., 2015; Simpson et al., 2013). In rural areas of Jersey and Channel Islands (Blackett et al., 2018) and Anglesey in Wales (Shuttleworth et al., 2015), only 2% and 20% of red squirrels (*Sciurus vulgaris*), showed signs of unspecific respiratory disease, respectively. On the Isle of Wight, Cumbria, Scotland, and Jersey, 35.2% of red squirrels showed pulmonary lesions associated with protozoan infection (Simpson et al., 2013). However, in the rural population of the Finlayson's squirrel (*Callosciurus finlaysonii*) localised lung disease was found in 69% of individuals, but no evidence of respiratory diseases, which was attributed to infection (Latta et al., 2015). Therefore, further research is required to understand the relationship between infection and TRAP exposure in the development of lung disease in urban squirrel populations.

Conclusions

As urban areas expand and encroach on wildlife habitats, the impact of urban stressors such as air pollution on wildlife health is becoming more apparent. In this study, we show that urban populations of grey squirrels are exposed and respond to air pollution and have a high prevalence of respiratory diseases. However, more, larger-scale and long-term studies are needed to understand the exposure to specific air pollutants, and differences in toxicity, as well as assessing a wider range of potential responses to air pollution and disease outcomes across a wider range of organs.

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Competing interests

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.

~~Conclusions~~

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