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Elevated lead (Pb) in urban European starling (*Sturnus vulgaris*) feathers is not correlated to physiology or behavior

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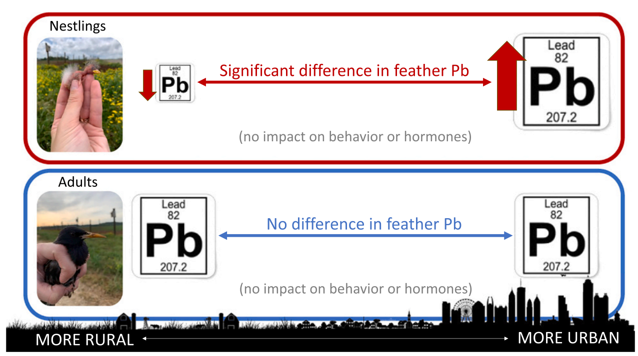
HIGHLIGHTS

- There was no difference in soil Pb between two urban and two rural sites in metro-Atlanta.
- Adult starlings from urban versus rural habitats had similar feather Pb concentrations.
- Nestlings from urban habitats had higher Pb concentrations in their feathers.
- Physiology and behavior did not correlate with feather Pb concentrations.
- Feathers of nestlings may be a possible non-invasive biomonitoring tool of Pb.

GRAPHICAL ABSTRACT



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ABSTRACT

Urbanization is rapidly changing the environment and creating new challenges in the lives of animals across the globe. Anthropogenic contaminants—like heavy metals—can persist within the environment for prolonged periods of time and present a widespread problem for those living near contaminated areas. Lead (Pb) was a commonly used heavy metal that continues to threaten the health of all organisms despite being phased out, especially in urban areas where historical use was more common. In this study, a common urban-adapter, the European starling (*Sturnus vulgaris*), was trapped to explore whether feather Pb burden is greater in birds from urban habitats than rural habitats, as well as whether Pb burdens were correlated with behavior, physiology, and feather development. Across four sites (two rural and two urban), soil Pb concentrations were measured and 197 free-living starlings were captured to measure feather Pb concentrations. Using linear mixed models, this study found that urban starling nestlings had elevated feather Pb burdens compared to rural nestlings. In contrast, there was no correlation between Pb and urbanization in adult birds whose exposure to Pb may reflect a larger spatial range compared to nestlings. For both nestlings and adults, feather Pb was uncorrelated to corticosterone, testosterone, aggressive behavior, or feather growth rates. These findings suggest that starlings may be a useful biomonitoring tool to detect Pb in the local environment, however, the age and spatial range of birds is a critical

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consideration in applying this tool. Further work is needed to understand the intricate relationship between heavy metals, behavior, morphological development, and physiology in free-living organisms.

1. Introduction

As urbanization increases globally, urban areas are encroaching on habitats needed by wildlife and impacting ecosystems. Human construction associated with urbanization has led to a marked increase in pollution and the impacts of pollution on organisms are complicated and not fully understood (Seress and Liker, 2015). One class of environmental contaminants that are very toxic at low doses are heavy metals (Miranda et al., 2013). Heavy metal contamination influences the integrity of the ecosystem by accumulating in soil, water and eventually in animal tissues leading to sub-lethal poisoning or death, which ultimately alters animal populations and fitness (Millaku et al., 2015). Heavy metals in water or soil do not degrade and can remain undetected for prolonged periods of time (Briffa et al., 2020). Lead (Pb) is one heavy metal contaminant that was historically commonly used in a variety of products. Despite Pb emissions decreasing due to the phase-out of Pb products in the 1990s, historical Pb emissions from as early as 1970 may still be present in the environment today while remaining undetected (Datko-Williams et al., 2014). Due to the fine-particle, aerosol nature of Pb, it can easily wash away from Pb-laden materials or structures into the environment (Mielke et al., 1984). Current industrial activities and traffic emissions continue to emit Pb in the environment today (Jeong, 2022; O'Shea et al., 2020; Sevik, 2019). Since Pb was commonly used in several construction and automobile materials, scientists hypothesize that Pb contamination increases in areas that are more urban. In support of this idea, studies have discovered that soil Pb is found in higher concentrations in more urbanized areas compared to rural ones and soil Pb is positively correlated to human density (Datko-Williams et al., 2014; Grue et al., 1986; Mielke et al., 2011; Pouyat et al., 2015). As a result, organisms living in urban areas may be at greater risk of Pb exposure and may accumulate heavy metals in their tissues more rapidly or at higher concentrations.

In order to identify areas with elevated Pb, it is typical to rely on environmental sampling (Peters et al., 2023) or human blood Pb screening (Centers for Disease Control and Prevention, 2006) (but see recent socioeconomic modeling approaches; Distler and Saikawa, 2020). Because environmental Pb is distributed unevenly, environmental sampling of soil, water or air can miss hotspots of Pb to which humans, domesticated animals or wildlife can become exposed. Furthermore, screening efforts are often concentrated around obvious contamination sites such as industrial plants (McLaughlin et al., 2000), and broader sampling is not always feasible or prioritized (Beardsley et al., 2021). An alternative approach is to use a biomonitoring tool—an organism or its tissues that can reflect the state of the environment—to identify areas with elevated Pb. A useful biomonitoring tool would be easy to collect, would reflect environmental exposure risk, and could be used across large geographic scales. Because they can be sampled non-invasively, bird feathers have been proposed as a biomonitoring tool and have led to the detection of previously unknown heavy metal sources within soils (Abbasi et al., 2015). Birds that forage in the soil are exposed to environmental Pb and uptake it orally or via soil deposits on their feathers. Non-migratory birds with known foraging ranges that can be clearly defined can reflect environmental Pb within their range (Durkalec et al., 2022), providing information about a wider area of land than one soil sample alone. Species that are widespread and accessible are a better choice for use as a bioindicator (Cai and Calisi, 2016). Feathers have the advantage of being easy and non-invasive to collect and can even be collected without capturing the bird (e.g., molted feathers). The limitations of biomonitoring tools include that they require validation, can be context dependent, and are not a direct measure of environmental Pb.

Monitoring environmental Pb exposure is important because of its known negative effects on vertebrates. From laboratory studies, the negative effects of Pb exposure on physiology, behaviors, and morphological development have been well documented in multiple vertebrate species (Assi et al., 2016; Dumitrescu et al., 2014; He et al., 2020; Kumar et al., 2020). In birds, it was shown that Pb affects hormone levels, critical early learning and survival behaviors, and morphological development—especially in reproductive organs (Burger and Gochfeld, 1988, 1995; Eeva et al., 2014; Leidens et al., 2018; Williams et al., 2017). However, these studies do not wholly reflect the real world and how organisms interact with their environment because researchers experimentally manipulated Pb dosages. The lethal and sub-lethal effects of Pb exposure in free-living organisms are complex—where animals are exposed to environmental Pb naturally rather than fed or injected with experimental doses—despite this being a critical question in the fields of urban ecology and conservation and the focus of many prior studies. For example, because sub-lethal effects could take time to accumulate and for negative impacts on fitness to manifest, some negative effects of Pb on organismal behavior, physiology, and development are difficult to monitor (Buekers et al., 2009). In a recent study, McClelland et al. (2019) observed a sub-lethal effect of Pb exposure in northern mockingbirds (*Mimus polyglottos*): they compared birds living in neighborhoods with low Pb soil concentrations to ones in high Pb soil concentrations and found that birds exposed to higher Pb concentrations had elevated concentrations in their tissues and responded more aggressively to perceived threats. In another study that compared common blackbirds (*Turdus merula*) living across different degrees of urbanization, trace element contamination burden increased with urbanization which resulted in endocrine disruption (Meillère et al., 2016). Both of these studies used feathers to measure Pb concentrations and infer the impacts of environmental Pb contamination.

One concern for pollutants that are endocrine disruptors—such as Pb—is that they can negatively impact the way an organism behaves in the face of different stressors in their environment and thus impair their ability to cope with non-pollutant challenges. Two major vertebrate endocrine systems that influence an animal's behavior and fitness—the hypothalamic-pituitary-adrenal (HPA) axis and the hypothalamic-pituitary-gonadal (HPG) axis—may be disrupted by heavy metals. The HPA axis regulates vertebrate responses to environmental changes and acute stressors (Majer et al., 2019), including urbanization (White et al., 2022). The HPA axis, through a series of intermediaries, controls circulating glucocorticoid (CORT) concentrations. Glucocorticoid hormones restore homeostasis following exposure to a stressor and regulate energy, immune reactions, and behavioral responses for animals exposed to environmental stressors (Sapolsky et al., 2000). When animals face a sudden stressor, CORT concentrations rise rapidly—called the stress response—which allows the individual to alter their behavior in order to overcome the stressor. Currently, it remains unclear the extent to which Pb exposure may disrupt the HPA axis and CORT due to differences in sampling methods (Meillère et al., 2016), though prior research found that nestling birds with higher Pb exposure showed increased CORT levels (Eeva et al., 2014). In addition, previous research suggests that the effect of Pb on CORT may vary between species or phase of the annual cycle (Provencher et al., 2016). In contrast, the HPG axis is primarily responsible for regulating reproductive activity through releasing reproductive hormones (Couse et al., 2003; Vadakkadath Meethal and Atwood, 2005), and mediating trade-offs between mating and parental efforts (Chatelain et al., 2018). Testosterone (T) is a reproductive hormone used to initiate reproduction, courtship behavior, and territorial aggression, and thus often shapes reproductive success (Chatelain et al., 2018). Previous research has shown that Pb exposure may increase T levels which can result in increased aggression in birds

(Wingfield, 1984, 1985) and humans (Wright et al., 2008). Although some studies have shown reproductive impairment from Pb exposure (Ding et al., 2019), the underlying physiological mechanism linking Pb and hormones remain unclear and one possibility is that these effects are mediated through disruption of the HPG axis.

It is essential to understand the impacts of Pb on these endocrine systems, because they shape a number of important behaviors when animals face a sudden challenge or during breeding, which could in turn affect reproductive success and fitness outcomes. For animals living across an urbanization gradient, adjusting behavior correctly based on the conditions that they face may be particularly important. It was expected that higher CORT and T would correspond with increased aggressive behaviors due to coping with the stressors related to sub-lethal Pb poisoning. In humans, the sub-lethal effects of Pb exposure can lead to antisocial behaviors such as aggression as well as increased physiological stress (Ahamed and Siddiqui, 2007; Wright et al., 2008). Similarly, researchers observed increased aggression post sub-lethal Pb exposure in laboratory animals (Cervantes et al., 2005; Delville, 1999; Li et al., 2003). The relationship between CORT and T levels and aggression are less clear in free-living birds. Although aggression in both sexes is thought to be shaped in part by T, there is some disagreement amongst the literature exploring these relationships in free-living birds (Fokidis et al., 2011; Ketterson et al., 2005; Lipshutz and Rosvall, 2021; Rosvall, 2013). Similarly, there is no consensus regarding the relationship between aggression and CORT levels (Davies et al., 2018; Fokidis et al., 2011). Nevertheless, understanding this relationship is critical for urban conservation.

This study investigated the effects of Pb exposure on a common songbird—the European starling (*Sturnus vulgaris*)—which breeds across an urbanization gradient. Adult and nestling starlings were captured to explore whether urban and rural birds differed in their Pb burdens. Feathers are as a less invasive method of monitoring Pb in birds compared to collecting blood samples or organs for heavy metal analysis. Heavy metals accumulate in feathers at the time of feather growth when compounds circulating in the blood of the animal get passively deposited in the feather. As a result, the Pb burden in a feather may also be linked to the quality or growth rate of that feather. To date one study showed there is indeed a negative correlation between increased exposure to Pb and feather growth rates. In another urban-adapted species, the great tit (*Parus major*), researchers found that experimentally elevated concentrations of Pb decreased feather growth rate (Talloen et al., 2008). It was expected that growth rates in feathers of birds exposed to higher concentrations of Pb would differ.

Predictions included that urban study sites should have higher soil Pb concentrations compared to the rural sites. Following this pattern, urban starlings should have higher Pb burdens in their feathers, which would correlate with higher circulating concentrations of CORT and T, more instances of aggressive behaviors, and slower feather growth rates. In addition to improving our understanding of the sub-lethal ways in which Pb exposure may shape the lives of urban animals, motivation for this work included determining whether animals may serve as bioindicators of heavy metals to inform risk to all organisms within the environment (Cai and Calisi, 2016; Dip et al., 2001; Swaileh and Sansur, 2006). Though some studies suggest that environmental Pb is decreasing over time (Helander et al., 2019), Pb poisoning remains a real concern for both humans (US CDC Advisory Committee on Childhood Lead Poisoning Prevention, 2021) and animals (Finkelstein et al., 2023; Van den Heever et al., 2022). This work is timely because elevated Pb in the soils of urban gardens remains a concern in the metro-Atlanta area where this study takes place (Saikawa et al., 2023; Yao et al., 2023).

2. Materials and methods

2.1. Study species

European starlings (*Sturnus vulgaris*) are endemic to Europe and parts of Africa and Asia but are also very common in their introduced ranges which include the Americas, South Africa, Australia, and New Zealand. As cavity nesters, who often build their nest in human-made structures, they are a common pest found naturally across the urbanization gradient. In Georgia, starlings are permanent residents, though populations in more northern locations are migratory (Kessel, 1953). Starlings typically breed in the early spring and raise up to several broods of young. During the breeding season, starlings mostly limit their home range to 500 m from their nest (Feare, 1984) where they forage in grass pastures using their beaks to probe the upper layers of the soil for soil- and ground-living invertebrates which they bring back to the nest to feed their young. Throughout the rest of the year, starling diets mostly consist of insects from the upper layers of the soil though they are omnivorous and consume a diversity of foods (Dunnet, 1955). Due to their ground foraging lifestyle, starlings are at greater risk of being exposed to Pb contamination in the soil (Pouyat et al., 2015).

2.2. Study area

This study was conducted in the metro-Atlanta region which is a sprawling urbanized area where the population between 2020 and 2022 has grown by 124,130 people; it is predicted to grow by an additional 2.9 million people by 2050 (Atlanta Regional Commission, 2022a, 2022b). Study sites were selected that had no known history of heightened Pb emissions but represented urban versus rural sites. Study sites included two rural locations: a cattle farm in Taylorsville, GA (34°05′50.7"N 84°54′10.8"W; average population density = 65.22/km^2) and a rural park in Cartersville, GA (34°07′47.5"N, 84°49′41.0"W; average population density = 289.71/km^2). Data were also collected at two urban sites: an old concrete plant that was converted to an urban farm in Acworth, GA (34°03′43.1"N 84°36′13.5"W; average population density = 732.4/ km^2) and an urban sports complex in Kennesaw, GA (34°00′12.6"N 84°37′07.7"W; average population density = 1312.88/ km^2) (Fig. 1). This study was conducted during the breeding season (March to June) in 2020–2022 across these four study sites. While both urban sites were monitored in all three years of the study, the rural cattle farm in Taylorsville was only monitored in 2020 and 2021, while the rural park in Cartersville was added in 2022 only. To compare the degree of urbanization between the field sites, 'UrbanizationScore' software was used (Lipovits et al., 2015) which scores the abundance of vegetation, buildings, and paved roads, ranking sites with a higher score as being more urbanized. This confirmed that the more rural sites differed from the more urban sites in their degree of urbanization (rural farm = −2.29, rural park = −2.01, urban farm = 2.13, urban park =2.17). Because the four study sites clearly clustered as two more rural sites and two more urban sites according to UrbanizationScore, they were classified by habitat type as 'rural' and 'urban' accordingly.

This study monitored nest boxes with active nests (n = 115) and captured 197 starling individuals (n = 62 adults, n = 135 nestlings). Adults were captured with a Van Ert spring trap at the nest box when nestlings were 3–10 days old, while nestlings were captured and sampled at the nest box at 16–17 days of age. During the 2020 breeding season, no adult starlings were sampled. Only adult birds were sampled from the urban park field site in Kennesaw as all nests found at this site were ultimately abandoned before nestlings reached sampling age.

2.3. Sample collection

When nestlings or adults were sampled, a baseline blood sample was collected from the brachial vein within 3 min of capture to measure baseline corticosterone (CORT) and testosterone (T) concentrations. Next, handling aggression was measured: holding the bird loosely, the observer placed the bird with its back on their palm and held the bird's head between their index and middle finger with the legs unrestrained. The observer then counted the number of struggles (e.g., kicking, scratching, biting) for 30 s. Following the handling test, birds were

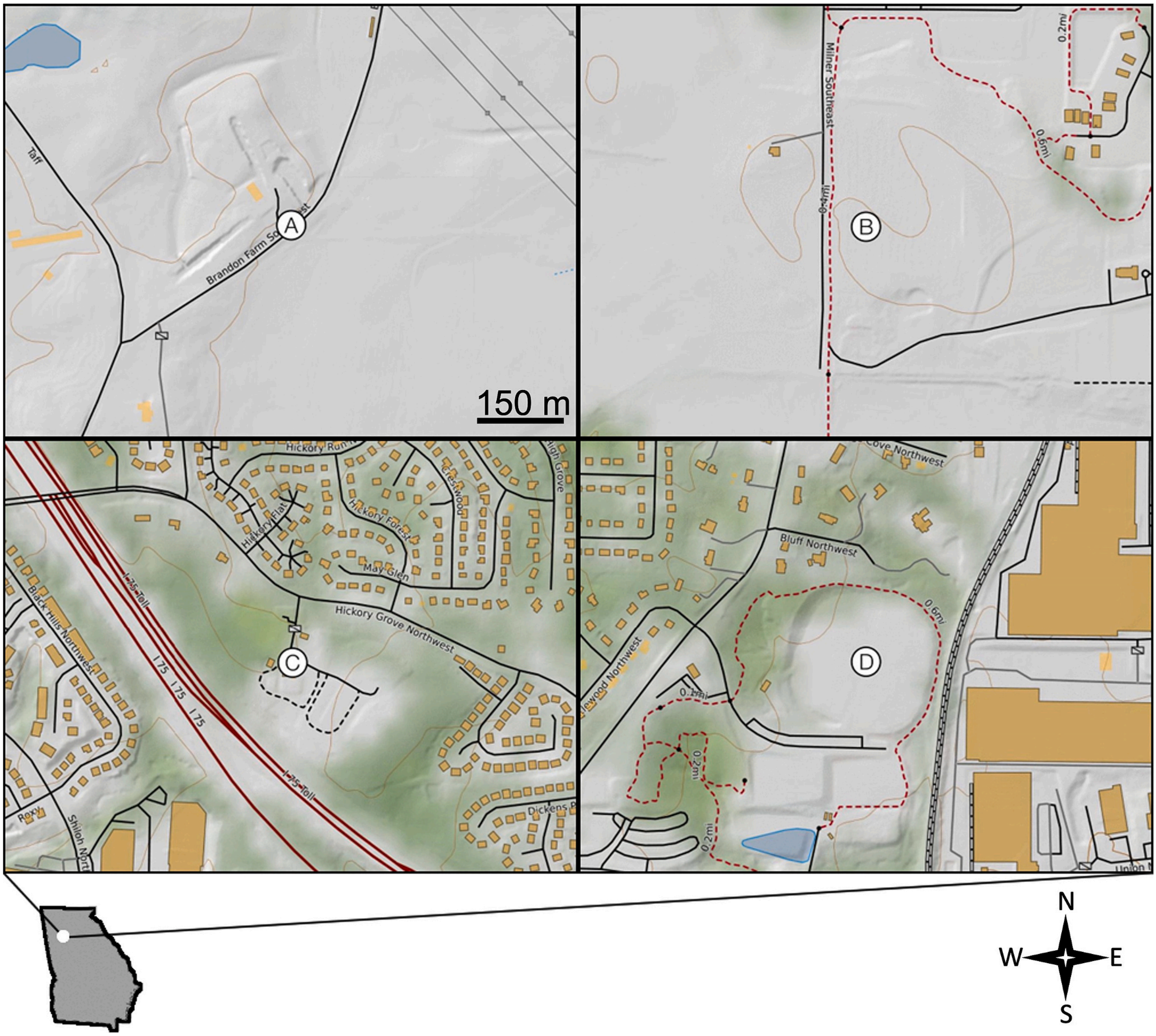

Fig. 1. Study sites in (A) Taylorsville, (B) Cartersville, (C) Acworth and (D) Kennesaw, Georgia. The orange shading represent buildings, the green shading represents tree cover, and the grey shading represents grass cover. Maps were generated using CalTopo.

immediately placed inside of a cloth bag and were suspended in the air by the observer. The observer then counted how many times the bird struggled for 30 s to assess isolated bag aggression. Both behavioral tests provide insight into the bird's response in a new situation (Guindre-Parker et al., 2022).

Following the isolated bag aggression, birds remained in the cloth bag. After 15 min for nestlings and 30 min for adults, an additional blood sample was collected to measure stress-induced CORT. Different time points were selected for nestlings and adults, because nestlings have been shown to attenuate their CORT stress response more rapidly than adults (Bebus et al., 2020; Guindre-Parker et al., 2022). Both blood samples for each individual were stored on ice until centrifuged (5 min at 10,000 rpm) within 4 h. Plasma was removed and stored the samples at −80 °C until further analysis.

The outermost right tail feather was plucked to assess feather growth rates and for Pb analysis. Adult starlings molt their feather during the pre-basic molt period after breeding once per year, sometime between June and September, and thus the feathers represent one year of Pb exposure in adult birds during the prior breeding season (Kessel, 1957; Rothery et al., 2000). In contrast, feather Pb represents primary exposure in nestlings who grow their tail feathers in the nest box of the current breeding season (Carlson et al., 2014). Feather growth rate was determined by measuring the total feather length (not including the pin) and dividing by the number of growth bars on the tail feather sample. Each growth bar, a dark and light bar visible on the feather, represents feather growth for a 24-h cycle (Brodin, 1993; Grubb Jr., 2006). Feathers were stored at room temperature until they were analyzed for Pb concentration by the University of Georgia's Agricultural and Environmental Services Laboratory (UGA AESL).

At each field site, a monthly soil sample was collected to assess soil Pb concentrations. The core sample were collected a depth of 25.4 mm and were 101.6 mm in diameter. This soil depth reflects the approximate depth at which starlings forage at and thus this is the layer of soil these birds would be routinely exposed to. Samples were stored at −20 °C until they were thawed and shipped to UGA AESL for analysis.

2.4. Glucocorticoid assay

CORT concentrations were quantified using a commercially available enzyme immunoassay kit from Arbor Assays (DetectX Corticosterone Enzyme Immunoassay Kit, K014-H5) following the manufacturer's protocol for small volumes and as previously validated for this species (Kilgour et al., 2022; Guindre-Parker et al., 2022). Each plate included a standard curve, ranging from 78 pg/ml to 10,000 pg/ml. 10 μl of dissociation reagent and 10 μl of plasma sample were combined together and set aside for 5 min. Then, 230 μl of assay buffer was added to each sample and 50 μl of the diluted sample was loaded onto a 96-well plate. Next, 25 μl of conjugate and 25 μl of antibody were added to all wells before incubating at room temperature for 1 h while shaking at 500 rpm. Plates were washed 4 times with 200 μl of wash buffer before 100 μl of TMB substrate was added to each well. The plate was left to incubate at room temperature in the dark for 30 min (no shaking). After 50 μl of stop solution was added, the plate absorbance was read using a BioTek plate reader (ELX808) at 450 nm and CORT concentrations were calculated based on the standard curve. Both baseline and stress-induced samples for the same individual were run on the same plate, but the individuals selected on each plate and the position of the samples on the plate were randomized. In addition, samples were assayed in duplicate. Intra-assay coefficient of variation (CV) was calculated by comparing duplicates of the same sample which was 5.14 %. In addition, the inter-assay CV was measured from a pooled plasma sample which was run on each assay plate. The mean inter-assay CV was 11.9 %. Baseline CORT and stress-induced CORT concentrations are expressed in ng/ml.

2.5. Testosterone assay

T concentrations were measured using a commercially available enzyme immunoassay kit from Arbor Assays (DetectX Enzyme Immunoassay Kit T Kit, K032-H5) according to the manufacturer's protocol. Prior to running the assay an extraction was performed as recommended by the kit. 40 μl of each plasma sample was combined with 200 μl of DI water at room temperature. Next, 1 ml of diethyl ether was added to each sample and tubes were vortexed for 2 min. The samples were flash frozen in a dry ice bath where the bottom layer (containing plasma and water) froze whereas the top layer (containing ether and T) was poured into a clean vial. T vials were left uncovered in a 30 °C water bath for 40 min to allow the ether to evaporate. Vials were capped and stored at −20 °C until the assays could be performed. In addition to each sample, extraction recovery was determined by using a T standard (10 ng/ml) run through the diethyl ether extraction protocol: briefly, 10 μl of the T standard was added to 200 μl of DI water before following the extraction procedure described above. The extraction efficiency was 134 ± 8.29 % which suggests high recovery of T from the extraction protocol (note that the kit's documentation also reports mean recoveries that exceed 100 %).

The T assay was run by creating a standard curve which ranged from 40 to 10,000 pg/ml. The extracted sample vials were brought up to room temperature and reconstituted them by adding 150 μl of assay buffer and vortexing each sample. 50 μl of standard and samples were added to wells in duplicate. 25 μl of conjugate was added, followed by 25 μl of antibody to each well and incubated the plate for 2 h at room temperature while shaking at 500 rpm. Plates were washed out 4 times with 200 μl of wash buffer and 100 μl of TMB substrate was added to each well. Next, samples were incubated at room temperature in the dark for 30 min (no shaking) before 50 μl of stop solution was added. Plate absorbance was read using a BioTek plate reader (ELX808) at 450 nm and T concentrations were calculated based on the standard curve. The mean intra-assay CV was 2.99 % and the mean inter-assay CV was 32.6 % (note that this includes variability caused by repeating the extraction for an aliquot of pooled plasma, as well as from running the same sample across different assay plates). T concentrations are expressed in ng/ml.

2.6. Lead assay

Individual tail feather samples and soil samples were analyzed for Pb at UGA AESL. Extraneous material like plants, rocks, and roots were removed from the soil samples. Feather samples were not washed prior to acid digestion—this means that feather Pb reflects both Pb ingested and deposited into the feather as well as surface Pb on the feather at time of sample collection. Including external contamination has been suggested to provide more information about the presence of Pb in the bird's environment (Jaspers et al., 2019; Borghesi et al., 2017; Borghesi et al., 2016). In addition, prior research has shown that vigorous washing is not effective in removing all exogenous contamination (Aloupi et al., 2020; Veerle et al., 2004; Dmowski, 1999; Weyers et al., 1988). At their facilities, both sample types were arranged in a tray and dried in an oven for ~24 h at 65 °C. Both sample types were passed through a 20-mesh screen Wiley mill and ground. UGA AESL uses the US-EPA Method 3052 (US-EPA, 1995) to digest samples. Solutions of each digested sample were analyzed for Pb using US-EPA Method 200.7 (US-EPA, 2001). All results are reported in percent or parts per million (mg/kg), with a detection limit of 0.005 mg/kg. Following US-EPA method 200.7, calibration blanks, independent calibration verification (ICV) and continuing calibration verification (CCV) samples, as well as standards from a certified source, were ran with each sample batch. Recoveries from ICV and CCV fell within 100 ± 5 %, and blanks were always 0.0 mg/kg.

2.7. Statistical analyses

Linear mixed effect models (LMM) were used to assess how (1) soil Pb concentrations differed across sites or habitat types (rural vs urban), as well as how (2) feather Pb concentrations were correlated with habitat type, feather growth rate, and behavioral and endocrine coping styles. All Pb concentration values were log-transformed to meet the assumptions of LMM. First, the model tested whether soil Pb concentrations differed across rural and urban sites: soil Pb (log) was the dependent variable and predictor variables included habitat type (classified as rural vs urban), year and Julian date of sampling. Nest ID was included as a random effect to account for non-independence of multiple samples collected beneath the same nest box.

Next, this study explored whether feather Pb concentrations varied with urbanization, feather growth rates, behavior, or hormones. One LMM was built for nestlings and one for adults, but with identical model structures. The dependent variable was feather Pb (log), and the following predictor variables: year of sampling, Julian date of sampling, mean soil Pb at site of sampling, urbanization status (rural vs urban), feather growth rate, handling aggression, isolated bag aggression, baseline CORT concentration, stress-induced CORT concentration, and T concentration. In addition, nest ID was used as a random effect to account for non-independence due to sampling both parents or multiple nestlings from the same nest. All analyses were performed in R (version 4.2.2; R Core Team, 2021). LMMs were performed in package nlme (version 3.1–160; Pinheiro et al., 2023).

3. Results

First, this study examined whether soil Pb concentrations differed across rural versus urban sites, expecting higher Pb in the soil of the urban sites compared to rural sites. The mean soil Pb concentration for the rural sites was 12.8 mg/kg ($n = 10$) and was 13.3 mg/kg for the urban sites ($n = 16$). The LMM revealed that the degree of urbanization did not correlate with soil Pb concentrations. Variance in soil Pb concentrations was high even within a site (Fig. 2). However, the Julian date and year the soil samples were collected were both significant predictors of soil Pb concentrations, with 2022 soil samples showing elevated Pb compared to 2021 and soil Pb decreasing as the breeding season progressed (Table 1).

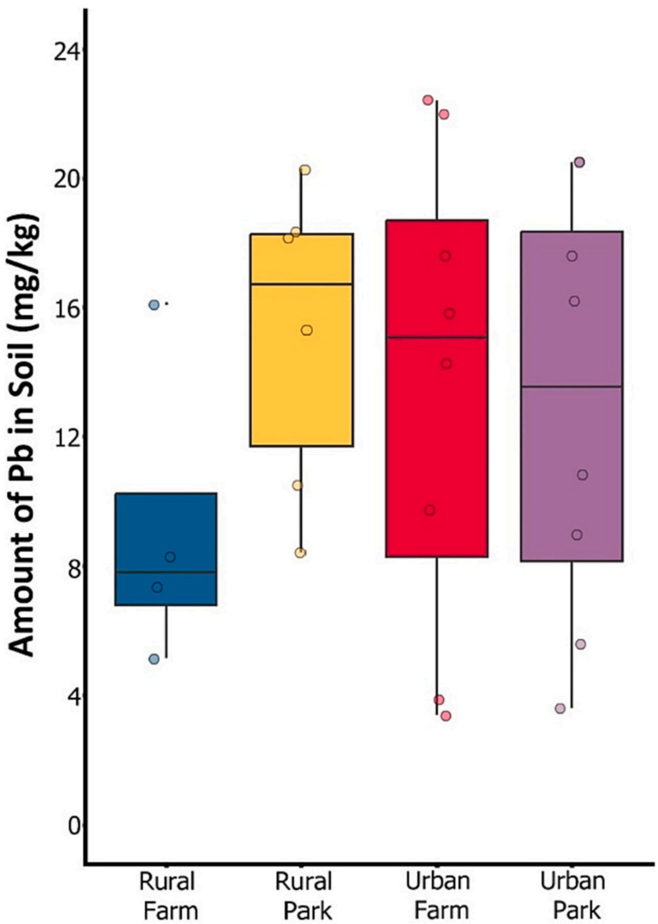

Fig. 2. Mean soil Pb from rural versus urban study sites. Each dot represents an individual data point (raw data). In these box plots, each whisker represents the 1st and 4th quartile, and the 2nd and 3rd quartile boxes are separated by the solid horizontal line which is the mean Pb concentration.

Table 1
Linear-mixed model (LMM) results comparing soil Pb concentrations to year, Julian date of collection, and urbanization status ($n = 26$). Asterisks and bolding denote significant predictor variables where *p*-value ≤0.05.

Fixed effects	Estimate ± se	t-Value	*p*-Value
Intercept	−2216.37 ± 367.02	−6.04	**0.000***
Urbanization status	−0.10 ± 0.16	−0.63	0.54
Year	1.10 ± 0.18	6.05	**0.000***
Julian date	−0.004 ± 0.0007	−5.20	**0.004***

Random effect	sdev
Nest ID	0.19
Residuals	0.31

Next, this study examined whether feather Pb concentrations differed between rural and urban starlings for both adults and nestlings separately. In the adult model, feather Pb was uncorrelated to any of the predictor variables, including year, Julian date, site type (rural vs urban), or soil Pb at the breeding site (Table 2). Adult feather Pb concentrations were also unrelated to circulating hormone concentrations—including baseline CORT, stress-induced CORT or T—as well as feather growth rates, handling aggression or isolated bag aggression (Table 2; see figures for non-significant correlations in supplementary materials). Mean adult feather Pb concentration was 1.99 mg/kg for rural starlings ($n = 52$) and 3.09 mg/kg for urban starlings ($n = 10$). In contrast, urban starling nestlings had elevated feather Pb concentrations compared to rural nestlings (Fig. 3). There was an effect of year on nestling feather Pb concentrations, where feather Pb appears to increase over the period of this study from 2020 to 2022 (Table 2). However, similarly to the adult starling results, nestling feather Pb concentrations were not correlated with Julian date or soil Pb at the breeding site. Finally, there were no significant correlations between nestling feather Pb concentrations and baseline CORT, stress-induced CORT, T, feather growth rates, handling aggression or isolated bag aggression (Table 2; see figures for non-significant correlations in supplementary materials). Mean nestling feather Pb concentration was 5.42 mg/kg for rural starlings ($n = 120$) and 10.45 mg/kg for urban starlings ($n = 15$).

Table 2
Linear-mixed model (LMM) results comparing Pb concentrations in feathers to date of collection, urbanization status, soil Pb concentrations, physiology, behaviors, and feather growth for (A) adults ($n = 62$) and (B) nestlings ($n = 135$). Asterisks and bolding denote significant predictor variables where p-value ≤0.05.

Fixed effects	A) Adults			B) Nestlings		
	Estimate ± se	t-Value	p-Value	Estimate ± se	t-Value	p-Value
Intercept	342.47 ± 934.97	0.37	0.72	936.94 ± 349.08	2.68	**0.008***
Year	−0.17 ± 0.46	−0.37	0.72	−0.46 ± 0.17	−2.68	**0.01***
Julian date	0.005 ± 0.006	0.70	0.50	0.009 ± 0.005	1.83	0.08
Urbanization status	0.16 ± 0.27	0.58	0.56	0.71 ± 0.33	2.11	**0.04***
Soil Pb	0.06 ± 0.08	0.68	0.50	−0.10 ± 0.05	−1.93	0.06
Baseline CORT	−0.01 ± 0.01	−1.16	0.28	−0.007 ± 0.004	−1.72	0.08
SI CORT	0.003 ± 0.004	0.60	0.56	0.002 ± 0.002	1.18	0.24
Testosterone	0.03 ± 0.09	0.36	0.73	−0.28 ± 0.25	−1.09	0.28
Feather growth rate	0.11 ± 0.24	0.47	0.65	0.09 ± 0.06	1.47	0.15
Handling aggression	−0.02 ± 0.03	−0.69	0.51	0.008 ± 0.01	0.71	0.48
Isolated bag aggression	0.01 ± 0.01	1.12	0.29	−0.006 ± 0.01	−0.51	0.61

Random effects	sdev	sdev
Nest ID	0.11	0.38
Residuals	0.44	0.47

4. Discussion

This study explored variation in soil and feather Pb across four study sites to determine if urban starling populations are exposed to higher concentrations of Pb and as a result, have higher amounts of circulating Pb within their tissues. Although there were no consistent differences in soil Pb associated with increasing urbanization, soil Pb concentrations did vary significantly from year-to-year and with Julian date. Due to the non-homogenous nature of soil or Pb deposits in the environment, Pb concentrations are likely to vary across soil cores, even when sampling the same area during different periods of time (IAEA, 2004). One explanation is that this study fell victim to the common pitfall that single soil samples do not reflect Pb exposure risk by missing hotspots of Pb—it is possible that urban habitats do indeed have elevated Pb exposure risk, which was not reflected in the soil samples in this study. To test this, future work could increase the number and coverage of soil samples in the future. An alternative explanation is that rural and urban sites have similar soil Pb concentrations as supported by this dataset. While many sources of Pb contamination are elevated in urban areas, rural habitats

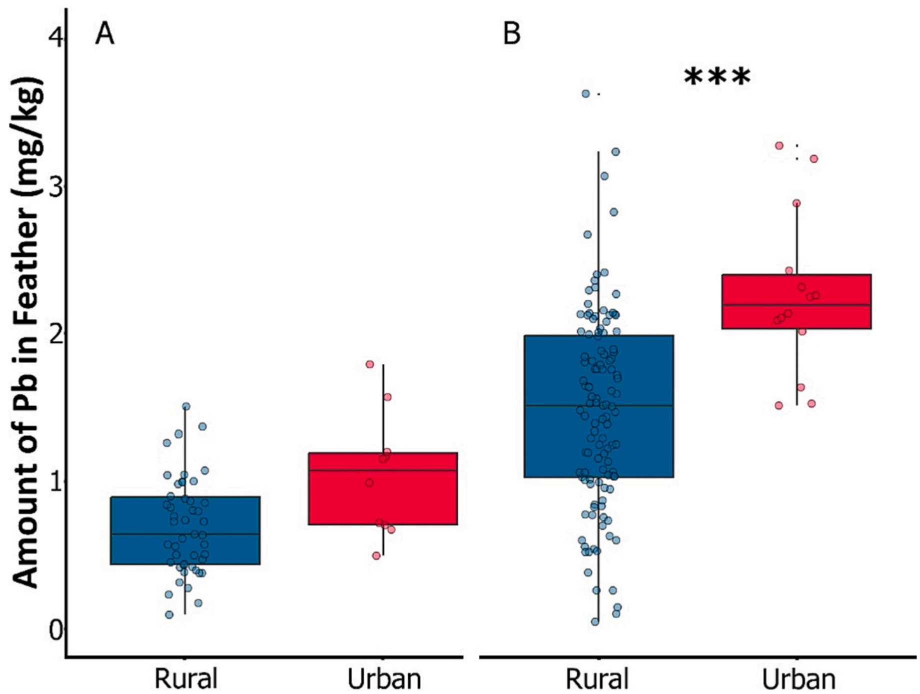

Fig. 3. Mean feather Pb concentrations for (A) adult and (B) nestling starlings from rural versus urban sites. Individual points indicate log-transformed data; the box and whiskers indicate quartiles and the solid horizontal line is the mean Pb concentration. Finally, asterisks indicate statistically significant differences amongst urban versus rural groups reported in Table 2 (p-value $\leq$0.05).

do have their own unique sources of Pb contaminants which may explain why some of the soil Pb datapoints from the rural sites are as high as in the urban areas. For example, rural farm machinery can release Pb. Similarly, Pb ammunition from hunting can pollute rural areas (Descalzo et al., 2021; Ecke et al., 2017; Vyas et al., 2000; Vallverdú-Coll et al., 2016). Elevated Pb can result near shooting ranges (Hardison Jr. et al., 2004; Lewis et al., 2001; Quy, 2010; Vyas et al., 2000), though there are no records of excessive shooting at the rural sites used in this study. Game birds and their scavengers are most at risk of Pb exposure via ammunition (Pain et al., 2009; Cardiel et al., 2011). While European starlings are unlikely to uptake Pb from ammunition, Pb emissions from farm equipment could be one explanation for the finding that soil Pb was similar in rural and urban habitats.

In contrast to the findings from soil samples, urban starling nestlings have higher concentrations of Pb compared to their rural counterparts which matches results from previous studies (Dauwe et al., 2000; Janssens et al., 2001; Janssens et al., 2003a; Roux and Marra, 2007). Additionally, although the result was not statistically significant, there was a weak pattern showing that nestling feather Pb concentrations increased with soil Pb concentrations at the site of their nestbox (p = 0.06). Taken together, this suggests that nestling feathers may be a useful bioindicator of environmental Pb which is of increased concern for urban dwelling populations. Future work using a larger sample size or sampling across a greater number of sites may better detect a correlation between soil Pb and feather Pb concentrations. In contrast to the findings in nestling starlings, this study found no differences in feather Pb concentrations between the rural and urban adult birds. Both adult and nestling mean feather Pb concentration results were similar to previous findings from other studies, with mean feather Pb ranging from 1 mg/kg to 7.75 mg/kg across different studies and different populations (Meillère et al., 2016; Scheifler et al., 2006; Fritsch et al., 2019). Similar studies in European starlings or blackbirds that compared urban and rural populations have also found that urban birds had higher feather Pb concentrations compared to their rural counterparts (Meillère et al., 2016; Scheifler et al., 2006; Fritsch et al., 2019). It is important to note that similarly to this study, feather Pb burdens from prior studies overlapped greatly across rural and urban sites—for example, mean rural feather Pb concentrations ranged from 1.00 to 4.27 mg/kg and mean urban feather Pb concentrations ranged from 1.00 to 7.75 mg/kg.

This work shows that Pb burdens appear higher on average in nestlings than adults, which supports prior studies showing that heavy metal burdens can differ between adult and juvenile life stages in birds (Lodenius and Solonen, 2013). Although adult birds might be exposed more directly to heavy metals than nestlings (i.e., by interacting with contaminated soil or water more directly), feathers of nestling birds appear to more accurately reflect the local pollution near the nest where they are raised and show similar Pb levels in some internal tissues due to being confined to the nest during development (Burger and Gochfeld, 1993; Franson and Pain, 2011; Janssens et al., 2003b; Janssens et al., 2001). Breeding starlings use a small range to forage for their young during nestling provisioning, which may lead the nestling Pb burden to reflect that local environment. However, since adults can experience a more extensive home range during the annual cycle—including after breeding when they molt their tail feathers—it may result in a weaker or absent correlation between adult feather Pb and environmental Pb at their breeding site the following year (Espín et al., 2016; Franson and Pain, 2011). Since feather Pb in adults reflects their exposure during the prior summer when they last molted their tail feathers, it is also possible that their current habitat does not reflect their exposure during the molt period if adults were to switch breeding sites across years. Another possible explanation is that there can be differences in Pb detected across different tissues and adult feathers are a poor indicator of current Pb exposure. For example, a study by Ek et al. (2004) showed heavy metals do vary in their distribution in different organs, feces, feathers, and eggs. They found that the concentration of Pb was significantly higher in kidney and blood samples compared to egg and feather samples. Different methodologies may give additional information about the adult populations that this study failed to detect (Lodenius and Solonen, 2013).

There was no correlation between elevated Pb levels and hormones in either adults or nestling birds. While nestling results suggest that a trend may exist between baseline CORT and feather Pb, there was no positive relationship between the two (p = 0.08). Although, there is some discrepancy amongst prior studies on whether increased Pb concentrations lead to increased hormone levels. Studies in similar urban-adapted songbird species showed a direct correlation between degree of urbanization, Pb and increased CORT concentrations (Bichet et al., 2013; Meillère et al., 2016). However, other studies on similar species showed no link between Pb and CORT nor T levels (Chatelain et al., 2018; Eeva et al., 2014; Provencher et al., 2016). No study has shown a positive correlation between urbanization, heavy metal exposure, and T levels in birds; the links between the HPG axis and heavy metals remain an understudied topic especially for free-living organisms. Overall, these results did not support that Pb exposure as detected in tail feathers was correlated with endocrine traits in starlings. Similarly, there was no correlation between elevated Pb concentrations and the assays of aggressive behavior. For nestlings, aggressive behavior may be limited in scope and occur primarily to compete with their siblings for food. Very low levels of T were present within nestlings which could drive low incidence of aggressive behaviors. However, another study in starlings did not detect a correlation between T levels and sibling competition (Gil et al., 2008). Aggression may be more important in adults than nestlings, when competition for mates, nesting sites, or towards predators may have greater fitness consequences. Adult birds were sampled during the breeding season when T concentrations and territorial aggression should be high. However, adult feather Pb was unrelated to aggressive behavior in starlings. In other urban-adapted adult birds, studies showed no correlation between aggression and T or heavy metal exposure during the breeding season (Davies et al., 2018; Grunst et al., 2018). Although McClelland et al. found a positive correlation between neighborhoods with high Pb concentrations and increased aggression in adult birds, this study did not measure T levels (McClelland et al., 2019). In a different study, researchers castrated male European starlings and found that although control males had higher levels of circulating T, the castrated males were significantly more aggressive which suggests that T levels and aggressive behaviors may occur independently of one another (Pinxten et al., 2003). These results suggest that contrary to the effects seen in humans, Pb as measured in feathers does not have a strong correlation with aggressive behavior in birds at the doses observed in this study. Finally, there was no correlation between elevated Pb levels and feather growth rate in either adults or nestlings. Previous research suggests that heavy metals, especially Pb, may impact the development of chicks who are exposed to high concentrations of heavy metals (Burger and Gochfeld, 1988; Spahn and Sherry, 1999). However, feather growth did not appear hindered in adults or nestlings with higher Pb burdens regardless of their site of origin. These findings are in agreement with prior research that failed to find any significant effects of heavy metal pollution on feather growth in free-living urban-adapted species (Dauwe et al., 2006). Although birds deposit heavy metals into their feathers, these results suggest that exposure to environmental Pb at the time of feather growth does not affect the development of the feather itself. Perhaps Pb in blood or tissues would be a stronger correlate of hormones or behaviors than feather Pb. Feather Pb concentration combines both Pb ingested and deposited in the feathers as well as surface contaminants—indeed this study found that feather Pb was higher than blood levels in starlings (mean $_{plasma}$ = 0.69 mg/kg, mean $_{feather}$ = 1.95 mg/kg for the same subset of 20 birds, *unpublished data*) (Espín et al., 2016; Franson and Pain, 2011). While feather Pb may represent a more useful biomonitoring tool than blood levels, blood concentrations may more directly correlate to the sub-lethal effects of Pb than feather levels. This could explain why there were no correlations between hormones or behavior and feather Pb in the present study. While feathers may be a stronger biomonitoring candidate than blood for ease of collection, feather Pb may be less informative about organismal health.

5. Conclusion

This study shows that urban starling nestlings have higher concentrations of feather Pb despite failing to detect soil Pb concentration differences between the field sites. These findings could indicate that environmental Pb is higher at the urban sites, and that nestlings are a stronger bioindicator of small-scale differences in environmental Pb than soil cores alone. In contrast, there were no relationship between rural and urban adult feather Pb burdens, suggesting that age class is an important consideration in using starlings as a Pb biomonitoring tool. Overall, non-invasively collected feathers from nestlings, but not adults, may be a useful biomarker of urbanization and Pb—because parents feed their young from a relatively small provisioning range during breeding and the nestlings grow their tail feathers over a few short weeks in the nest, the feather Pb of nestlings appears to be a better representation of the local environment than feather Pb in adults. While there were no correlations between starling feather Pb concentrations and hormones, behavior, or feather growth, future work incorporating longer-term measures of fitness would be essential to understand whether these different Pb burdens impact organisms. Experimental studies grounded in ecological ones—for example, studies using doses of Pb closer to concentrations detected in natural habitats—would be needed in order to better understand the sub-lethal impacts of Pb on behavior and physiology. This work suggests that incorporating feathers of urban-adapted birds as bioindicators of heavy metals may be a valuable addition to assessing environments compared with soil sampling alone.

CRediT authorship contribution statement

Michelle Ross: Conceptualization, Methodology, Investigation, Writing – original draft, Visualization, Funding acquisition. **Joanna L. Corimanya:** Investigation, Writing – review & editing. **Rachel Kaplan:** Investigation, Writing – review & editing. **Denyelle A.V. Kilgour:** Investigation, Writing – review & editing. **Courtney R. Linkous:** Investigation, Writing – review & editing. **Sarah Guindre-Parker:** Conceptualization, Methodology, Investigation, Writing – review & editing, Project administration, Funding acquisition.

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.

Data availability

Data attached as supplementary material csv files

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2023.168932.

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