Sub-lethal exposure to lead is associated with heightened aggression in an urban songbird

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Abstract

Many urban areas have elevated soil lead concentrations due to prior large-scale use of lead in products such as paint and automobile gasoline. This presents a potential problem for the growing numbers of wildlife living in urbanized areas as lead exposure is known to affect multiple physiological systems, including the nervous system, in vertebrate species. In humans and laboratory animals, low-level lead exposure is associated with neurological impairment, but less is known about how lead may affect the behavior of urban wildlife. We focused on the Northern Mockingbird *Mimus polyglottos*, a common, omnivorous North American songbird, to gain insights into how lead may affect the physiology and behavior of urban wildlife. We predicted that birds living in neighborhoods with high soil lead concentrations would (a) exhibit elevated lead concentrations in their blood and feathers, (b) exhibit lower body condition, (c) exhibit less diverse and consistent vocal repertoires, and (d) behave more aggressively during simulated conspecific territorial intrusions compared to birds living in neighborhoods with lower soil lead concentrations. Controlling for other habitat differences, we found that birds from areas of high soil lead had elevated lead concentrations in blood and feathers, but found no differences in body condition or vocal repertoires. However, birds from high lead areas responded more aggressively during simulated intrusions. These findings indicate that sub-lethal lead exposure may be common among wildlife living in urban areas, and that this exposure is associated with increased aggression. Better understanding of the extent of the relationship between lead exposure and aggression

and the consequences this could have for survival and reproduction of wild animals are clear priorities for future work in this and other urban ecosystems.

Highlights

- Sub-lethal lead exposure of urban wildlife is widespread, but effects are unknown.
- We evaluated exposure and behavioral correlates in the Northern Mockingbird.
- Birds in high lead areas had higher blood and feather lead and were more aggressive.
- No differences were observed in vocal repertoire or body condition.
- Behavioral consequences of lead exposure to urban wildlife deserve more attention.

1. Introduction

Lead (Pb) pollution remains a critical global issue despite recent curtailments on its use (World Health Organization, 2010). On account of the large-scale previous use of lead in gasoline, house paint, and other products, lead remains present in large quantities in many urban settings (Gulson et al. 1995, Callender and Metre 1997). The accidental ingestion and inhalation of lead in urban environments (Kennette et al. 2002, Beyer et al. 2013) can result in urban populations – humans and animals alike – with physiological lead concentrations several times higher than background levels (Roux and Marra 2007, Grue et al. 1986, Scheifler et al. 2006, Cai and Calisi 2016). Urban areas are home to a substantial and growing amount of the world's wildlife (Marzluff et al. 2008), whose conservation and evolutionary ecology is a burgeoning field of inquiry (Magle et al. 2012, Lowry et al. 2013). For these reasons, understanding patterns of lead uptake in urban wildlife and the consequences of exposure for their health represents an important goal for the scientific community.

Lead exposure and associated physiological and behavioral effects have been well studied in humans and laboratory animals (Needleman 2004, Smith et al. 2012) and lessons from this research can inform expectations for how lead exposure might impact urban wildlife. High doses of lead are often fatal (Doomeya et al. 2011, Grant 2008) and although lead shot is now banned for waterfowl hunting in the United States (Cruz-Martinez et al. 2012), millions of birds are believed to continue to die annually of acute lead poisoning though ingestion of lead-based ammunition and fishing weights (De Francisco et al. 2003). However, most cases of lead exposure among urban wildlife and pets are likely to be sub-lethal, and little is currently known about the impacts of sub-lethal lead exposure on wildlife, in particular the effects on behavior (Hunt 2012). This represents an important gap in our knowledge because, given the quantity of lead in the environment worldwide, several million wild animals and pets are likely exposed to sub-lethal levels of lead (Mateo-Tomás et al. 2016, Meyer et al. 2008, Omelchenko 2011).

Sub-lethal lead exposure may have irreversible effects on physiology, cognition, and behavior (Schwartz 1994, Burger 1990, Burger and Gochfeld 2005). Lead exposure in humans and laboratory animals, especially during early development, impacts the central nervous system (Flora et al. 2012, Stewart et al. 2006) and can result in mental retardation, hyperactivity, reduced IQ and difficulty with emotional processing (Chen et al. 2012, Sanders et al. 2009, Banks et al. 1997). Among humans, sub-lethal exposure to lead has also been linked to increased aggression, including violent crime (Stretesky and Lynch 2004, Mielke et al. 2011, Stretesky and Lynch 2001), and a number of studies have reported significant positive correlations between lead exposure and crime rates or teenage delinquency irrespective of many social and economic factors (Needleman et al. 2002, Stretesky and Lynch 2001). Similarly, increased aggression has also been reported from controlled exposure trials on laboratory animals (Delville 1999, Cervante et al. 2012, Burright et al. 1989). Delville et al. (1999) found lead exposed male Golden Hamsters (*Mesocricetus auratus*) showed more territorial aggression (i.e. were more likely to bite and attack intruders), a similar result to Holloway and Thor (1986) who found lead increased play-fighting among juvenile rats. Other studies have found either a reduction in aggression with lead intake or no significant change, which suggests behavioral changes may be dose and/or species dependent, and that additional research on these relationships would be useful (Abu-Taweel et al 2006).

Among wild populations, Janssens et al. (2003b) reported a mild effect of a combination of heavy metals, including lead, on territorial aggression in the Great Tit (*Parus major*) living close to a metallurgic smelter. More recently, Grunst et al. (2018) explored personality traits of Great Tits living along the same heavy metal gradient and found consistent differences in exploratory behavior, but no apparent effect on territorial aggression. Relatively more work has been done on physiological correlates of lead exposure among wildlife, with impacts that include compromised immune function (Vermeulen et al. 2015, Snoeijs et al. 2004, Vallverdú-Coll et al. 2015), reduced body condition (Hohman et al. 1990, Roux and Marra 2007, Janssens et al. 2003a) and impaired brain growth (Douglas-Stroebel et al. 2004). Addressing the gap in our collective knowledge about the impact of lead on animals in the wild is important because behavioral and/or physiological impairment could have substantial consequences for individual fitness and reproduction and, by extension, population health (Lowry et al. 2013, Alberti 2015). It also raises the possibility that exposure to lead could be an under-appreciated driver of observed behavioral differences between urban and rural individuals of a given species (see Lowry et al. 2013, Carrete and Tella 2011).

In the current study, we examined relationships between lead exposure and physiological and behavioral correlates in the Northern Mockingbird (*Mimus polyglottos*; hereafter 'mockingbird'), a widespread and iconic North American songbird (Stracey and Robinson 2012, Kaufman 2001). Mockingbirds serve as a useful model for our study because the species is common in urban and suburban areas, including across gradients of lead soil concentrations in many cities (Blair 1996, Derrickson and Breitwisch 1992). Moreover, mockingbirds exhibit life-long learning of song, and repertoire complexity and consistency is associated with reproductive success (Botero et al. 2009, Gammon and Altizer 2011, Howard 1974, Yasukawa et al. 1980). In other songbird species, song is a useful index of developmental stress (Peters et al. 2014, Nowicki et al 2002) and song complexity and consistency indicative of greater cognitive ability (Boogert et al. 2008, Boogert et al. 2011, Farrell et al. 2012). Additionally, the mockingbird's territorial behavior and restricted home range during the breeding season (Logan 1987, Derrickson and Breitwisch 1992) allow linkages between lead exposure and local soil concentrations. Breeding mockingbirds also exhibit conspicuous and easily observed aggressive behavior around the nest, allowing researchers to quantify intensity of aggressive response via experimental presentation of a standardized stimulus. Our hypothesis was that mockingbirds exhibit behavioral and physiological correlates with lead exposure similar to those reported in human and laboratory animal studies. We predicted that birds living in areas with high vs. low soil lead concentrations will exhibit (a) higher lead concentrations in their blood and feathers (b) diminished body condition, (c) less diverse and stereotyped vocal repertoires (an index of cognitive ability) and (d) behave more aggressively during simulated conspecific territorial intrusions.

2. Methods

2.1. Study site.

Three residential neighborhoods in New Orleans that vary in soil lead concentration (Mielke et al. 2016) and habitat "greenness", based on GIS analysis of satellite imagery (below), were selected for our study. We compared differences between neighborhoods rather than individual territories because lead concentrations are heterogeneous at fine spatial scales (Mielke et al. 2016) such as a mockingbird's territory; access needed to adequately sample representative lead concentrations across a given territory was often impossible due to private property concerns; and we were sometimes unsure as to the precise boundaries of a given bird's territory.

2.2. Soil lead estimation

Based on a published census tract-based survey of soil lead across New Orleans, we selected three neighborhoods that differ in lead content (Mielke et al. 2016, Laidlaw et al. 2017). Among these, Lakeshore (center: 30° 02'3441" N, -90°09'3301" W; 2-49 mg/kg) has average lead concentrations at least one order of magnitude lower than either Marigny (center: 29° 96'5263" N -90° 05'6102" W; 300-500 mg/kg) or Uptown (center: 29° 94'0932" N – 90° 12'6437" W; 300-500 mg/kg), which in turn have similar lead levels (Mielke et al. 2016; Figure 1). To corroborate the results of these earlier studies, we tested soil lead levels in situ in each neighborhood using a portable energy dispersive X-ray fluorescence analyzer

(EDXRF). Sixteen mockingbird territories were tested in both Marigny and Lakeshore neighborhoods, and 24 in the Uptown neighborhood. Seven measurements were collected at least 5 meters apart at each territory and an average per territory was taken from these measurements.

2.3. Greenness estimation

Breeding territories in rural Texas (the closest geographic proximity with documented breeding territory sizes) ranged from 0.66 to 2.53 ha (Howard 1974), which also corresponds with our estimates of territory size for a sub-set of banded individuals in New Orleans (J. Karubian unpublished). Thus, we approximated habitat cover for a mockingbird's territory from a radius of 45 and 90 m away from the point of the aggression assay. We used April 2015 National Agricultural Imagery Program (NAIP) imagery obtained from EarthExplorer (earthexplorer.usgs.gov). NAIP imagery is downloaded and georeferenced with a Ground Sample Distance of 1 m. Six separate NAIP images (adjacent files captured on the same date) were mosaiced together prior to image classification. Next, we used an interactive supervised classification in ArcMap 10.3 (ESRI, Redlands, CA, USA), classifying the landscape of New Orleans into four categories: open vegetation (e.g., grass, lawns), canopy cover, water, and urban (i.e., anthropogenic). Here, we present data only on the vegetation parameters of this analysis; although we calculated water and urbanization separately, the classification analysis often classified pixels known to be urban in nature (e.g., buildings, roads) as 'water' and vice-versa. However, because none of the buffers overlapped with a major water body, water per se is likely not a significant factor contributing to mockingbird behavior and we are confident that these pixels represent the urban landscape in our study system. We calculated percent cover of each habitat cover type using the 'Extract by Mask' tool for each 45m buffer. An average score of 'greenness' versus other habitat, was calculated for each neighborhood as calculated by the mean of the vegetation percentage cover for 16 to 24 mockingbird breeding territories within each neighborhood. This was considered a 'greenness' score for the neighborhood. Subsequently, neighborhoods were compared for greenness using a one-way ANOVA.

2.4. Study design.

We used a low-lead neighborhood with a high greenness score (Lakeshore) and two high lead neighborhoods, one with a high greenness score (Uptown) and one with significantly lower greenness than the other two neighborhoods (Marigny) (Figure 1.). In this study design, the traits of birds in Uptown (high lead / high greenness) are of critical importance. If Uptown birds more closely resemble birds from Lakeshore (low lead / high greenness) then greenness would be implicated as a likely causal factor in driving observed patterns of physiology and aggression. In contrast, if Uptown birds more closely resemble birds from Marigny (high lead / low greenness), then lead would be implicated as a likely causal factor in driving observed patterns of physiology and aggression.

2.5. Sample collection

Birds were captured during the breeding seasons of the years 2015, 2016 and 2017 between the months of March and July. Adult birds (n = 34; 14 from 2015, 8 from 2016, and 12 from 2017) were captured using walk-in traps, mist-nets and foot-noose traps, typically with the aid of playback recordings of male territorial songs. Upon capture, a small blood sample was taken (~150 µl) via brachial venipuncture for blood lead analysis and genetic sex determination (from birds captures 2015 and 2017 only, n=26). Samples were stored in clay sealed heparinized capillary tubes at 4°C pending analyses. The third secondary feather from each wing was collected by plucking for feather lead analysis. Birds were banded with uniquely numbered aluminum band and three plastic colored bands for field identification. Standard morphometric measurements were collected, including weight, tarsus length (measured twice and averaged to reduce variation), wing and tail length, culmen and molt scores. After processing, all birds were released at the point of capture.

2.6. Lead analysis

Samples of blood and feathers collected in 2015 (blood n=14, feathers n=14) were analyzed by Activation Laboratories Ltd. (Ontario, Canada), whereas sample collected during the 2016 and 2017 field season (blood n=12, feathers n=16) were analyzed at the Microbiology and Environmental Toxicology department in the University of California, Santa Cruz as described below.

2.6.1. 2015 sample analysis

Feathers. To remove external contamination, samples were washed twice with deionized water followed by rinsing in 1 mol/L acetone (Optima grade, Fisher Scientific) and then placed in a 45 °C oven for 4–5 h until completely dry, at which point they were weighed. Samples were digested with HNO_3 (Optima grade, Fisher Scientific) and H_2O_2 (Ultra grade, Fisher Scientific) and twice heated at 85 °C in a JULABO hot water bath (Allentown, PA).

The ratio of HNO_3 to H_2O_2 was approximately 3:1. Whole blood. Digestion and analysis of blood followed the same procedure as for feathers, except the ratio of reagents used to digest the blood was 1:1 instead of 3:1. Lead analysis. Samples were diluted to 10 mL with deionized water, and concentrations were measured with a Thermo Scientific ™ iCAP Q inductively coupled plasma-mass spectrometer (ICP-MS). Samples were spiked with internal standards (iridium and rhodium) and analyzed in batches with certified reference material (SRM 1575a) from the National Institute of Standards and Technology (Gaithersburg, MD). Recoveries ranged from 85 to 110%.

2.6.2. 2016 /2017 sample analysis

Biological (blood and feather) samples were processed and analyzed using established trace metal clean techniques and ultra-pure reagents, as described elsewhere (Finkelstein et al., 2010; Finkelstein et al., 2003; Gwiazda et al., 2005; Smith et al., 1996).

Feathers: The entire feather was washed sequentially with acetone, ultrapure water, 1% HNO_3 and ultrapure water to remove surface contamination. Feathers were then dried overnight at 60°C, weighed, digested overnight in 2 mL sub-boiling concentrated HNO_3 (optima, Fisher Scientific) in closed Teflon vials, evaporated to dryness, and reconstituted in 5% HNO_3 for analysis. Whole blood: Blood (~5-70µL) was transferred from heparinized capillary tubes into trace metal clean micro-centrifuge tubes, weighed, then dried overnight at 60°C to obtain dry weight values, and digested as follows: 100 µL (for samples with blood volume < 50 µL) or 150 µL (for samples with blood volume > 50 µL) of concentrated HNO_3 (optima, Fisher Scientific) as added to each sample and samples were digested cold for 10 hours. 30% H_2O_2 (ultrex, JT Baker) and ultrapure water was added to each sample for a HNO_3 to H_2O_2 ratio of

2:1 and approximate final concentration of HNO_3 of ~6%. Samples were vortexed and left to sit overnight before analysis. Lead analysis: Lead concentrations were determined by inductively coupled plasma mass spectrometry (ICP-MS, Finnigan MAT Element magnetic sector), measuring masses of ^{208}Pb and ^{205}Tl (used as an internal standard). Approximately 20 µL of NIST SRM 955c (lead in blood, level 2) was digested using the methods described above for blood with an average recovery (n = 3) of 99.6% ± 7% RSD. Capillary tubes (n =3) had an average processing blank of 0.002 (0.001 - 0.002) total ng lead, which was subtracted from all blood lead data (lowest blood was ~0.3 total ng lead).

2.7. Body condition

Body condition was calculated using the ratio of the mass to tarsus length (weight/tarsus), a commonly used measure of condition (Johnson et al. 1985). 28 birds were included in body condition analysis, excluding a small number of captured birds (n=6) for which weight or tarsus length was missing.

2.8. Song recordings and analyses

Male songs were recorded during an 18-day period, between 2 and 20 March 2015, in the mornings (7:30-12:00), soon after sustained male singing was observed across territories. We concentrated the period of recording to as few as possible days after onset of singing to control for nesting stage, which may affect certain song characteristics (Derrickson 1988), and alternated recording days between both sites. Based on population level data we have recorded (Karubian, unpublished data), it is likely that most if not all birds were in the nest initiation stage, but we were unable to verify nest stage for these birds due to constraints associated with working in an urban environment.

We did not attempt to capture males prior to audio recording, thus song and blood/feather samples are not available for the same individuals. Since males were not individually marked during the recording phase, sampling was spread geographically (minimum distance ~200 meters) within each study area to avoid pseudo replication. Recordings were taken only in one high lead neighborhood (Marigny) and the low lead neighborhood (Lakeshore). These neighborhoods were visited on alternate days and searched for singing males. Recordings were made as close to the bird as possible (4-20 m) using an omnidirectional Sennheiser ME62 microphone equipped with a windshield, mounted on a Sony parabolic dish, and connected to a Marantz PMD661 MKII digital recorder (.wav files, 44.1 khz, 16 bits/sample,). We recorded each focal male for as long as possible, interrupting and reinitiating recording if the bird moved or paused singing.

For each focal male, one ~3min long clip of continuous singing was analyzed using RavenPro v1.5 software (Bioacoustics Research Program 2013). Only high quality recordings were used, with high signal-to-noise ratios and without loud background, overlapping sounds. Clips were bandpass filtered to include only sounds with frequencies between 1.3 and 10 khz, which include the bird songs but exclude much of the low-frequency background urban noises. Within each clip, individual syllable types were identified and counted. A syllable was defined as a sound, or group of sounds, that was separated from other sounds by more than 0.04 s of silence (Botero et al. 2009). A particular song can be composed of one or more syllables. Despite the large diversity of syllable types found in clips, classification of syllables was straightforward because of the repetitive nature of mockingbird songs, where a particular song is repeated a few times before switching to a new one. All quantifications of song parameters were made separately for each male, and no effort was made to quantify similarities in syllable or song composition between different males. Syllable classifications for all clips were made blind with respect of the areas where the recording was performed and were conducted by one of two independent observers and repeated or checked by Renata Durães Ribeiro (RDR).

Two measures of syllable versatility were quantified, following Derrickson (1988): syllable type versatility (number of syllable types divided by the total number of syllables contained per clip) and syllable switch rate (number of transitions between two different syllable types divided by the total number of possible transitions). Syllable consistency was measured as the average spectral cross-correlation (SPCC) among different renditions of the same syllable, following Botero et al. (2009). Twenty syllable types were randomly selected per individual, and 3-10 repeats were used per syllable type (depending on available number of renditions in clip), with every repeat being compared to every other repeat of the same syllable type. Peak correlation values were calculated between spectrograms based on linear power and normalized to vary from 0 (no similarity between the two sounds) to 1 (sounds are identical). To minimize the influence of background noise, only the frequency bands covered by each syllable type were used in the comparisons. SPCC coefficients were averaged for each syllable type, then across the 20 syllable types, to provide a mean syllable consistency coefficient for each focal bird. Syllable type versatility, switch rate, and syllable consistency were compared for adult males recorded in low or high lead areas using Mann-Whitney tests. In most cases, the recordings used to estimate syllable versatility were the same used to estimate syllable consistency; in three cases, however, recordings made for a given male were too short to estimate versatility, or not clean enough to estimate consistency. As such, the identity of individuals used in these two analyses differed slightly.

2.9. Aggression trials

Presentation experiments designed to measure aggression towards conspecifics by free-flying birds with active nests were conducted between 25 March and 29 June 2016 and between 19 May and 10 July 2017, periods that coincided with active breeding in the three neighborhoods. 24 presentations took place in the Lakeshore neighborhood, 21 in the Marigny Neighborhood and 38 in the Uptown neighborhood. Lakeshore and Marigny presentations were undertaken in the 2016 field season, and the Uptown presentations in the 2017 field season. As soil lead levels are believed to be relatively consistent over multiple years, we did not consider an effect of year of sampling on our results (Meilke et al. 2016).

In each trial, a simulated territory intrusion was staged using a taxidermized mount of a mockingbird accompanied by playback of a singing male. The taxidermized mount was posed in an aggressive posture (wing patches exposed and tail raised), placed in a protective cage atop a tripod at a fixed height of 1.5m and covered with a cloth until the beginning of the aggression trial. The auditory stimulus consisted of recorded songs of singing males played on a portable Pignose® speaker placed directly beneath the mount. Recordings were made by RDR in 2015 from territories in both high and low neighborhoods (to account for possible difference in song quality depending on neighborhood; henceforth, 'stimulus type'), and had their RMS amplitude normalized to 3,000 units in RavenPro v1.5 prior to use in experimental trials. Four high-quality recordings of similar duration from each neighborhood were used as stimuli and birds were exposed to a randomly selected stimulus from either neighborhood.

In each trial, the mount was placed in an open area at a standardized distance of 8 m from active nests during the period of nest construction and before the start of incubation to control for potential differences in response intensity associated with nesting stage. Before starting the trial, the focal, free-flying bird was determined to be within line of sight of the mount, at which point the trial began by uncovering the mount and playing 1 minute of recorded song at 85 dB from a speaker placed under the mount. This recording was repeated for 30 seconds at 3 additional time points (02:30 min, 05:30 min, 07:00 min) during the 10-minute trial. The bird was considered to have responded to the experimental intrusion when it moved toward the mount; latency to response was recorded as the time elapsed between the start of the trial and this initial response. If no birds responded during the 10-minute period, the trial was not included in our analysis in order to eliminate false zeros and the presentation was not repeated towards this bird. This situation occurred only in a very small number (<5) of attempted presentations and did not appear to be influenced by neighborhood.

Over the 10 minute trial, the distance in meters between the focal bird and the mount was recorded at the start of every minute. If more than one free flying bird responded, the locations and activity of both birds were recorded and the individual with the stronger response was considered to be the male of the pair as males participate in territory defense more frequently than females (Breitwisch et al. 1986). Aggressive displays were recorded continuously during the trial and included: number of “hissing” scold calls; number of times wings were raised to display wing patches; number of times tail was raised; singing (number of minutes); number of swooping flights towards the mount (fly-bys). If the bird landed on the cage, total time on the cage (in minutes) was recorded. Only one trial was conducted per pair / nest to control for potential pseudo-replication.

2.10. Data analyses

Data were analyzed using one or more statistical methods, as appropriate for the given data type. Lead concentrations of blood and feathers were analyzed using one-way analysis of variance (ANOVA) with neighborhood identity (Marigny, Uptown or Lakeshore) as a fixed effect. Pearson's product-moment correlation between blood and feather samples was calculated for individuals. One-way ANOVA's were similarly used to compare greenness scores and lead values between neighborhoods. The results of the above one-way ANOVA were also confirmed using Multi-Response Permutation Procedures (MRPP). A two-way ANOVA was used to analysis body condition. Neighborhood and sex of the adults (when known, n=14 of 28), and their interactions were included as predictor variables. The results of the aggression trails were analyzed using a combination of Principal component analysis (PCA) and MRPP, described in detail below. ANOVA and PCA were performed in R Studio 1.1.419 (RStudio Team 2015). Tests in R Studio were two-tailed. Values represent means ± 1 S.E. MRPP tests were run using the Blossom Version W2008.04 statistical package (Cade and Richard 2005). Effect sizes were calculated using the package sjstats in R studio (Lüdecke D 2018)

2.10.1. Principal component analysis

As several of the aggressive response variables were correlated with each other, we performed a Principal Component Analysis (PCA) to generate composite measures of aggressive response. Response variables included in the PCA were number of minutes perched on the mounts cage, number of minutes within 5 m of mount, number of scold calls, number of tail raises, number of wing raises, number of fly-bys, and latency to respond to the presentation. The first two principal components were used as response variables when comparing birds from high and low lead neighborhoods using ANOVA models. In addition to neighborhood, the stage of the nest (before or after laying of the first egg; all trials were conducted prior to the onset of incubation, which begins after the final egg of the clutch is laid), stimulus type (song from high or low lead area) and the presence or absence of females at the nest were considered as predicator variables in initial models. The best models for the first and second PC response were selected through stepwise model reduction (removing non-significant variables) based on AIC score. Only neighborhood was included as a predicator variable in the final model for both PC responses.

2.10.2. Multi-Response Permutation Procedure

Multi Response Permutation Procedures (MRPP) are a group of Euclidean distance-based statistical tests that make no assumption about the distribution of the data and satisfy the congruence principle. The probability value (p-value) associated with the MRPP is the proportion of all possible test statistic values under the null hypothesis that are less than or equal to the observed test statistic of the actual observations. (Cade and Richards 2005, Mielke PW, et al. 2017, Berry et al. 2014).

2.11. Study design limitations

2.11.1. Limitations of blood lead measurements.

Due to the relatively small size of mockingbirds (49.7g ± 4.3) blood was collected into a heparinized capillary tube as noted above. Capillary tubes were then stored up to several months at ~4°C until processing. Many blood samples were partially dried and thus wet weights from these samples are not reflective of the original blood volume collected into the capillary tubes. Blood lead values from samples collected in 2017 are reported in µg/dL based on dry weight values with a correction factor of 0.2, calculated from the wet weight: dry weight ratio of NIST SRM 955c samples (n = 3, 0.19 – 0.21). Blood lead values from samples collected in 2015 (no blood samples were analyzed from 2016) were reported based on wet weights. Reported lead concentration values for the mockingbirds in this study should be interpreted given these sample collection and storage limitations. The potential error in the blood lead measurements reported is not expected to alter the overall trends in differences in lead levels in Mockingbirds between neighborhoods, but should be noted for future comparisons with other studies on blood lead concentrations in avian species.

2.11.2. Natal dispersal and movement

In contrast to the inferences we can make about the apparent lack of seasonal movements in this population (above), we are at present unable to make any inferences about the extent of natal dispersal and therefore we do not know the degree to which individuals that were nestlings and fledglings in a high lead neighborhood subsequently dispersed into a low lead neighborhood, or vice-versa. This represents another important area for future inquiry, because of the disproportionate importance that exposure to contaminants during early development may have on behavior and cognition (Burger 1990, Burger and Gochfeld. 2005). Additionally, data for this study were collected over three consecutive breeding seasons between the years 2015 and 2017. While we believe soil lead exposure was consistent over this period, inter-annual variations in weather, food availability or competition may have existed which we would be unable to account for. Mean precipitation and temperature for these months did not differ substantially from recorded seasonal averages from the past century (NOAA National centers for Environmental Information), but it is still possible that there may have been variation for which we were unable to control.

3. Results

3.1. Soil lead

EDXRF measurements of soil lead taken across the three focal neighborhoods confirmed the differences in lead concentration previously reported in Mielke et al. (2016). The results of the MRPP analysis on soil lead by EDXRF found that Lakeshore had significantly lower lead than Marigny (Median lead concentration: Lakeshore = 7 mg/kg versus Marigny = 264 mg/kg, $p < 0.001$) and Uptown (Median Lead concentration: Uptown = 112 mg/kg, < 0.001). No statistical difference in soil lead was detected between Marigny and Uptown ($p = 0.139$), consistent with Mielke et al. (2016). The effect sizes of the differences between Marigny and Lakeview and between Uptown and Lakeview were substantial ($\eta_p^2 = 0.89$ and 0.30

respectivley) with 95% confidence intervals not crossing zero in either case. Whereas between Uptown and Marigny the effect size was small (η_p^2= 0.11) and the 95% confidence intervals crossed zero, suggesting little difference in soil lead concentrations.

3.2. Lead levels in blood and feathers

Lead levels among adult mockingbirds were higher in both the high lead neighborhoods than in the low lead neighborhood, for whole blood samples (low lead: Lakeshore = 3 ± SE 0.6 µg/dl , n = 7, high-lead : Uptown = 10 ± SE 2 µg/dl, n =12, Marigny = 10 ± 2 µg/dl, n = 7; $p < 0.001$, F_{11} =16.5 ; Fig.3a) and feather samples (low lead: Lakeshore= 2.76 ±SE 1.28 µg/g, n=8; high lead: Marigny = 13.19 ±SE 1.97 µg/g, n=12, Uptown= 14.20 ±SE 1.74 µg/g, n=10 ; $p < 0.001$, F_{27} = 10.7, Fig. 3b).

MRPP analysis corroborated these results, finding equally significant differences in both blood (Table. 2.a) and feather (Table 2.b) lead levels between high and low lead neighborhoods.

3.3. Body condition

There was no significant difference in the body condition of birds between neighborhoods (mean ± SE: Lakeshore = 1.36 ± 0.29, n =7, Marigny = 1.29 ± 0.02, n =12; Uptown = 1.37 ± 0.04, n = 9; F_{20}=1.5, $p = 0.23$), nor was there any significant effect of sex (1.34 ± 0.08 males, 1.38 ± 0.19 females, F_{20} = 0.39, $p = 0.67$), or the interaction term (F_{20} = 0.85, $p = 0.48$). There was no correlation between individual body condition and blood lead ($R = 0.327$, $p = 0.13$, df= 20) or feather lead ($R = 0.07$, $p = 0.72$, $df = 23$).

3.4. Song quality

Males in the low (Marigny, n = 5) or high (Lakeshore, n = 7) lead neighborhoods did not differ in syllable type versatility (low lead: 0.25 ± 0.13 syllables types/total number of syllables; high lead: 0.19 ± 0.04; $Z = 0.81$, $p = 0.42$) or syllable switch rate (low lead: 0.41 ± 0.12 transitions/total number of possible transitions; high lead: 0.39 ± 0.08; $Z = 0.16$, $p = 0.87$). Males in low or high lead areas also did not differ in syllable consistency (low lead: 0.79 ± 0.02 average spectral cross-correlation; high lead: 0.84 ± 0.02; $Z = -1.62$, $p = 0.10$).

3.5. Aggression trials

3.5.1. Principal component analysis

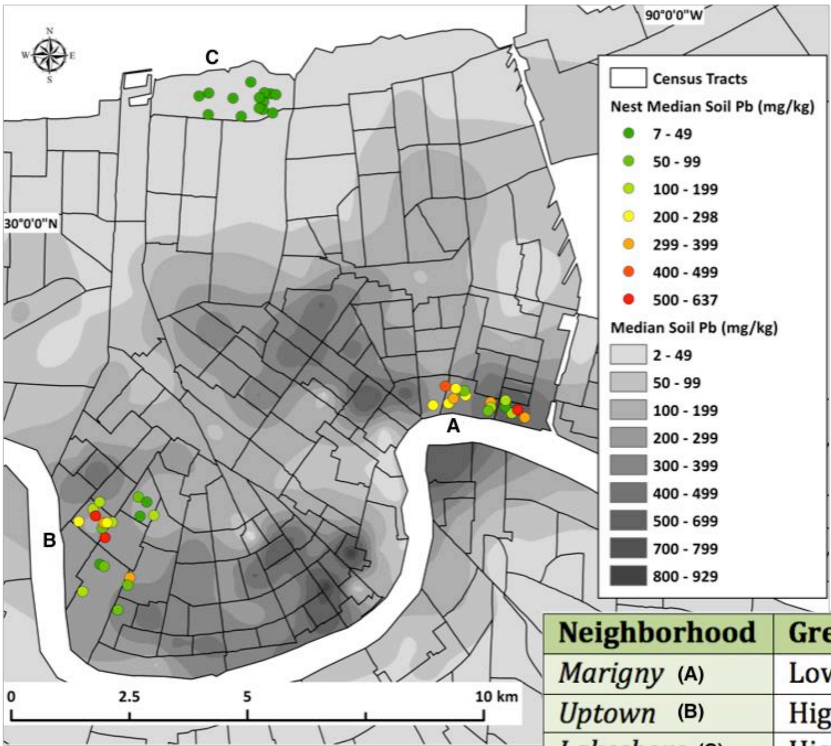
The first two principal components explained 51% of the variance in aggressive response of the birds (Table 1). PC1 was influenced mainly by number of minutes within 5 m of mount, number of scold calls, number of tail raises, number of fly-bys and latency to respond; PC2 was dominated by number of wing raises and number of minutes on the cage. Thus, higher values of PC1 and PC2 were associated with a more aggressive response.

PC1 was significantly higher in both high lead neighborhoods ($p < 0.001$, $F_{80} = 12.5$, Figure 4) compared to the low lead neighborhood (Lakeshore), but the high lead neighborhoods did not differ significantly from each other (Marigny and Uptown, $p = 0.08$). PC2 did not differ significantly between neighborhoods ($p = 0.09$, F_{80}=2.39), but there was a non-significant trend for lower values in Lakeshore.

3.5.2. MRPP

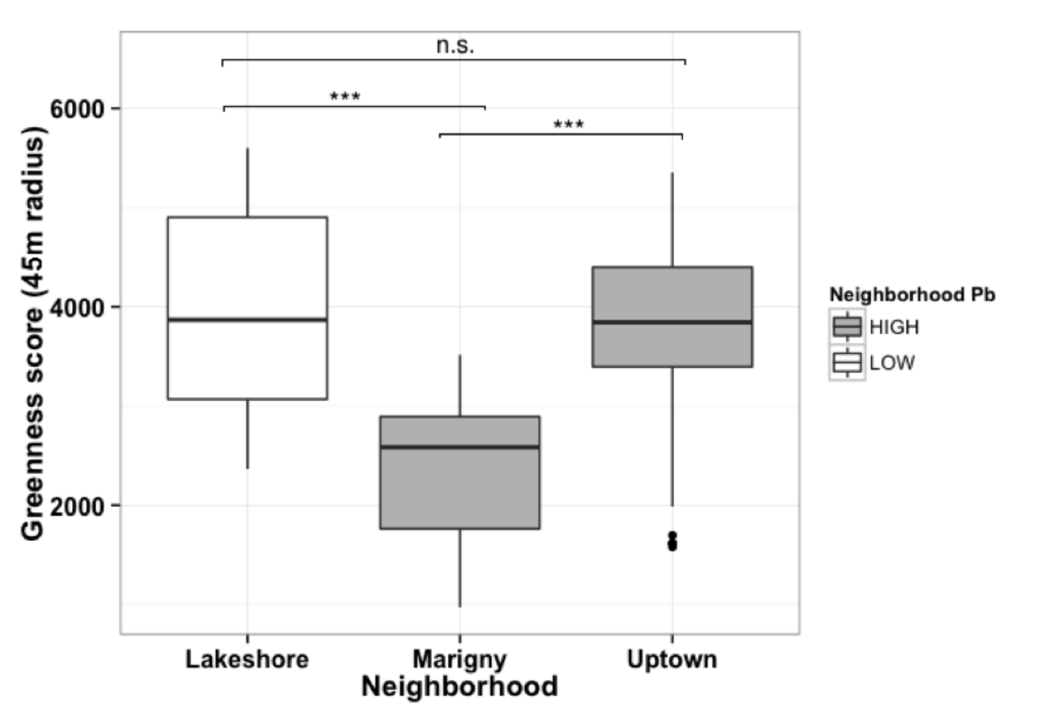
The results of the MRPP analysis were in agreement with the PCA analysis, with significantly higher rates of scold calls, fly-bys and tail raises (which was described by PC1) in the high lead neighborhoods (Marigny and Uptown) compared to the low lead neighborhood (Lakeshore) (Table. 3, supplementary material).

Figures



Neighborhood	Greenness	Soil Lead
Marigny (A)	Low	High
Uptown (B)	High	High
Lakeshore (C)	High	Low

Figure 1. Soil lead concentrations of New Orleans sampled between 2013 and 2015. Study sites are depicted by bold black letters, A) Marigny (High-lead, Low-Green), B) Uptown (High-lead, High-Green) and C) Lakeshore (Low-lead, High-Green),. Grey-scale represents soil lead medians presented in Mielke et al. 2016. The MRPP results indicate extremely small p-values (< 0.0001) for soil lead between C compared with A and B, while A and B have similar amounts of soil lead. Very large differences in greenness (i.e. p-values < 0.0001) appear between A compared with B and C, while greenness of B and C are similar. Greenness and soil lead categories show in the green text box.



<u>Figure 2.</u>

Greenness score of territories by neighborhood, based on 45m radius around nests (n=83). Marigny differs significantly from both Lakeshore and Uptown (*p<0.001*); there was no significant difference between Uptown and Lakeshore (*p=0.64*). Comparisons in greenness score between Marigny and Lakeview and between Marigny and Uptown had a large effect sizes (η_p^2 = 0.40 and 0.26 respectively) with 95% confidence intervals not crossing zero, whereas between Lakeshore and Uptown effect size was relatively low (0.11), with 95% confidence intervals encompassing zero.

a) b)

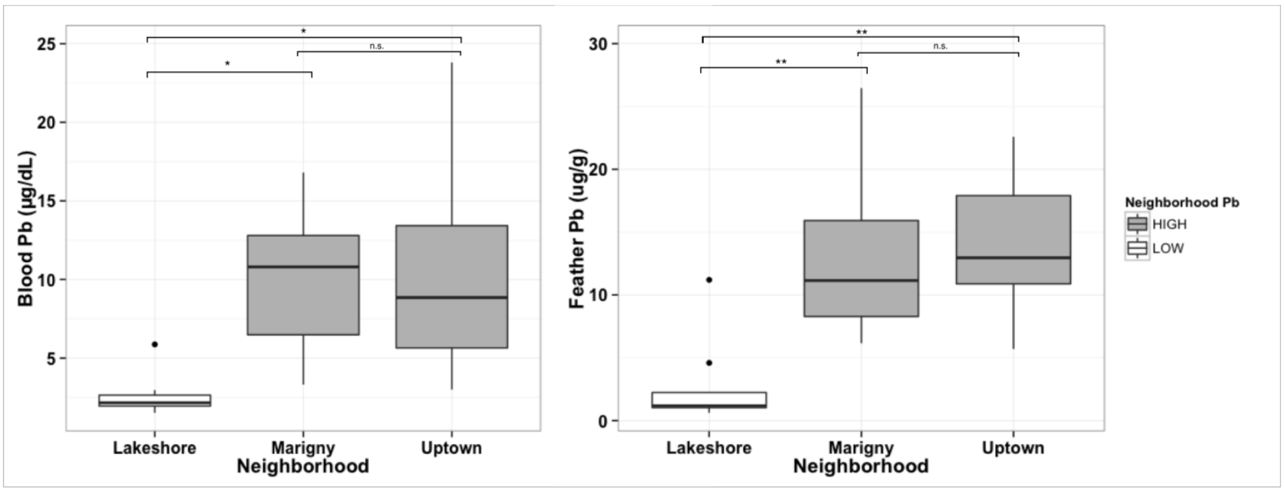


Figure 3. Blood (a) and feather (b) lead (Pb) concentrations (µg/dL and µg/g respectively) documented in northern mockingbirds in three New Orleans neighborhoods. Northern mockingbirds in the high lead neighborhoods (Marigny and Uptown) had higher concentrations of lead in the blood and feathers relative to birds from the low lead neighborhood (Lakeshore) (** = <0.01, * = <0.05).

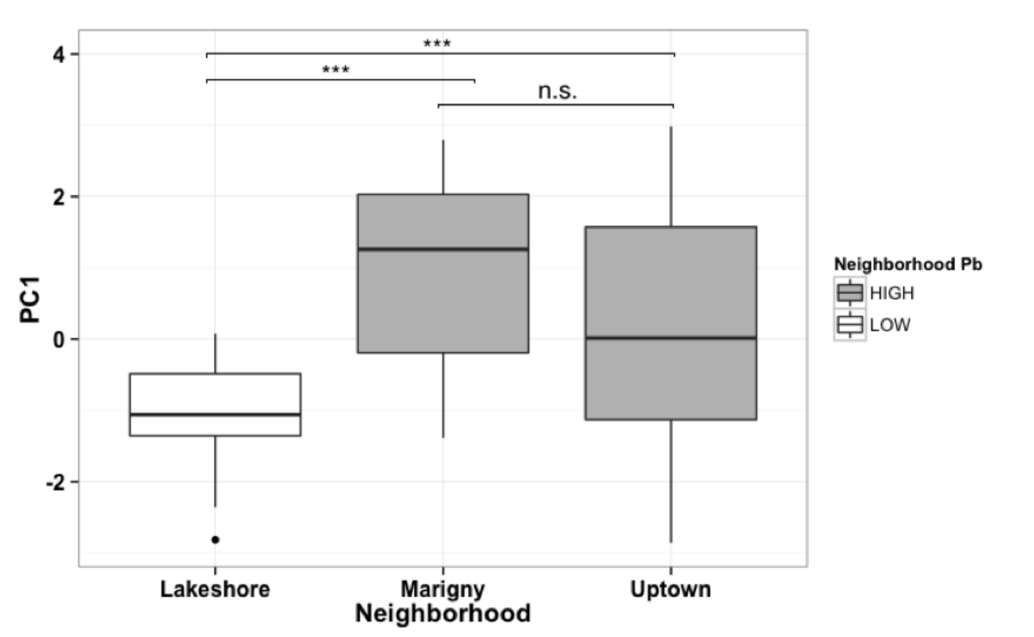


Figure 4. First principal component (PC1) representing aggressive response by adult breeding northern mockingbirds from three neighborhoods differing in soil lead levels, in response to a simulated territorial intrusion. Northern mockingbirds in the high lead neighborhoods (Marginy and Uptown) exhibited a more aggressive response to stimulated intrusions than did birds from the low lead neighborhood (Lakeshore). (*** = < 0.001) . Statistical significances presented in figure are from pairwise comparison of neighborhoods. PC1 was dominated by

number of minutes within 5 m of mount, number of scold calls, number of tail raises, number of fly-bys and latency to respond.

Table 1

(a) Principal Components Analysis (PCA) of aggressive response by breeding male northern mockingbirds to a simulated territorial intruder. (b) Eigen factor loadings from PCA of aggression trial responses. Factor loadings for principal components > |0.50| are in boldface.

(a) Proportion of variance explained

	PC1	**PC2**	**PC3**
Proportion of Variance	0.33	0.17	0.15
Cumulative Proportion	0.33	0.51	0.65
df	83	83	83

(b) Eigen loadings

Min on cage	0.44	**-0.76**	-0.14
Min < 5m cage	**0.71**	0.46	-0.16
No. Scold	**-0.56**	0.18	**0.58**
No. Fly By	**0.78**	-0.04	0.05
No. wing raise	0.27	-0.32	**-0.79**
No. tail raise	**0.55**	**0.52**	0.03
Latency	**0.54**	0.10	0.21

Table 2.

Lead concentrations in blood µg/dL (a) and feathers µg/g (b) of Mockingbirds captured in different neighborhoods, and *p* values from MRPP comparisons between neighborhoods. Median values shown in blue/italic.

a)

Blood Pb in µg/dL	**Lakeshore**	**Marigny**	**Uptown**
n	7	7	12
min	1.5	3.3	3
25%	1.9	5.6	5
50%	**2.2**	**10.8**	**8.9**
75%	3	5.6	13.7
max	5.9	16.8	23.8

MRPP Comparisons	**Test Statistic**	***p* value**
Lakeshore Vs Marigny	-4.93	0.003
Lakeshore Vs Uptown	-5.38	0.002
Marigny Vs Uptown	0.92	1

b)

Feather Pb in µg/g	**Lakeshore**	**Marigny**	**Uptown**
n	8	12	10
min	0.6	6.2	5.7
25%	0.9	8.2	10.2
50%	**1.2**	**11.2**	**13**
75%	3.8	16.8	19.2
max	11.2	26.5	22.6

MRPP Comparisons	**Test Statistic**	***p* value**
Lakeshore Vs Marigny	-7.17	2.1×10^{-4}
Lakeshore Vs Uptown	-7.34	3.3×10^{-4}
Marigny Vs Uptown	0.47	0.586

4. Discussion

Our findings are consistent with a strong correlative relationship between soil lead levels, corresponding concentrations of lead in blood and feather, and intensity of aggressive response among free-living individuals of an urban songbird, the Northern Mockingbird. There was no indication that any difference existed between neighborhoods in vocal repertoire and complexity, which we used as an indirect metric of potential developmental stress and cognitive ability in this species, or physiological condition.

4.1. Environmental, blood and feather lead

Adult birds from neighborhoods with high soil lead had on average four times more lead in blood and feathers and were significantly more aggressive in the context of simulated territorial intrusion. Importantly, differences in aggressive response tracked environmental lead levels rather than neighborhood greenness, a proxy for habitat quality (Gaston 2010, Shih 2017).

To put our blood lead results in perspective, the blood lead concentrations of adults from our high lead neighborhoods were on average ten times the level that has been linked by some studies to neurological impairment in children (Canfield et al. 2003). Further, Cervantes et al. (2012) found that golden hamsters doubled the rate of aggressive behaviors when fed a lead-supplemented diet resulting in blood lead concentrations of 5.5 ± 07 μg/dL, which is approximately half the average concentrations found in the blood of mockingbirds in our high-lead neighborhood (10 ± 2 μg/dl). The concentrations we recorded are all the more striking given the fact that soil lead levels in our two 'high' lead neighborhoods are in fact quite moderate relative to many urban areas (Laidlaw et al. 2017). Although limited evidence suggests that birds may be able to tolerate higher blood lead concentrations than mammals before showing adverse effects (Scheuhammer 1987, Buekers et al. 2009), health effects, such as reduction in essential enzyme functioning, have been documented in bird species with similar blood lead concentrations to those we report here (Pain 1989, Work and Smith 1996).

4.2. Aggression

We used an experimental approach based on presentation of a taxidermic conspecific mount (i.e., stimulus) to free-flying birds in the nest construction phase to evaluate one such effect: increased aggression. We found that high lead birds exhibited increased frequency of calls and aggressive displays and spent more time in close proximity to the stimulus relative to birds from the low lead neighborhood. This pattern was robust across two methods of data analysis and mirrors findings previously observed in humans, where the relationship between lead exposure and aggressive antisocial behavior has been robust across several studies (Dietrich et al. 2001, Mielke and Zahran 2012, Stretesky and Lynch 2001, Weber et al. 2013), and in captive animals, where several studies have found similar relationships (Li et al. 2003, Delville 1999, Cervantes et al. 2005). However, there are few comparable studies we are aware of in wild birds. These include Janssens et al. (2003b) who found only minor differences in the responsiveness of a territorial great tits (*Parus major*) from areas contaminated with heavy metals, including lead, towards a simulated intrusion, and Grunst et al 2018 who found no effect of proximity to a heavy metal source on aggression, though found other personality traits to be affected. Thus, there is evidence to suggest that the impacts of lead may vary across species or contexts, highlighting the need for additional work on this topic from a broader array of organisms.

It is not clear how this apparent increase in aggression may influence survival and reproduction in northern mockingbirds. On the one hand, hyper-aggressive behavior could reduce reproductive success, or survival, or both via heightened energetic demands (e.g., vigorously responding birds exhibited symptoms of physical stress such as bill gaping; SMC personal observation). For example, it might be associated with reduced vigilance and its associated increases in susceptibility to predation (Dunn et al. 2004, Hess et al. 2016), or time budget trade-offs that serve to reduce foraging efficacy and offspring provisioning. On the other hand, increased aggression could increase fitness by increasing the ability to defend territories and secure resources and mates through competitive interactions (Smith and Blumstein 2008). Additionally, lead-induced aggression could have implications for cuckoldry and extra pair paternity rates by influencing the intensity of interactions with intruding males, or alternatively weakening pair bonds if intra-pair aggression occurs (Westneat and Stewart 2003; Moreno *et al.* 2010). Further work to better understand the fitness consequences of lead-induced aggression in this system would improve our collective understanding of the consequences of chronic lead exposure among urban wildlife. Likewise, further research is needed to establish the mechanism behind increased aggression in lead exposed birds, such as how lead impacts on neurological function might interact with endocrine regulated behavior.

4.3. Mockingbirds as an indicator of environmental lead

In contrast to studies on species whose movements are unknown or that may be relatively mobile (e.g., urban pigeons (*Columba livia)* with a home range of ca. 2 km; (Cai and Calisi 2016)), mockingbirds are strongly territorial during the breeding season, when the current study was conducted, and, in many cases, year-round (Logan and Wingfield 1990, Logan 1987), strengthening the putative linkage between local soil and organismal lead levels. As suggested by Cai and Calisi (2016) in regards to urban pigeons, the ability to link lead levels in mockingbirds to local environmental lead levels suggests that this species may be useful as a bio-indicator of lead contamination risk for other species, including humans, associated with given areas. Blood lead is reflective of lead exposure over several days prior to sampling, whereas feather lead reflects exposure over the longer period of feather growth prior to molt (Burger 1993). In mockingbirds, molt occurs in August - October of each year, approximately nine months prior to our data collection period (Zaias and Breitwisch 1990), Farnsworth et al. 2011). The fact that lead levels in blood and feathers showed a similar trend in relation to neighborhood lead, therefore suggests relatively stable exposure across these time periods, consistent with limited seasonal movements and perhaps year-round territoriality in our study population.

4.4. Song performance

Motivated by literature linking song performance to cognitive ability in birds (Pepperberg 2009, Boogart et al. 2011a), we predicted that birds in high lead neighborhoods would exhibit reduced singing ability as a result of cognitive impairment caused by lead exposure. However, a recent study on captive swamp sparrows (*Melospiza georgiana*) found no relationship between song performance and cognitive ability (DuBois et al. 2018). No difference between adults from high vs. low lead neighborhoods were found in the traits we measured, suggesting that lead may not impact cognitive function as assessed by song quality. As such, more direct tests of cognitive ability (e.g. problem solving ability or utilization of novel resources; e.g., (Seed et al. 2006, Grodzinski and Clayton 2010, Clayton et al. 2001) may be needed to more resolve the relationship between cognitive ability and lead exposure in this system. Additionally, due to the large variation between individuals and relatively low sample size of this study, it is possible that our analysis lacked sufficient power to pick up potentially subtle difference between neighborhoods.

4.5. Body condition

There was no significant difference in our measure of body condition between individuals from high vs. low lead neighborhoods, although there was a non-significant trend toward adults in Marigny, (the high-lead, low-greenness neighborhood) having inferior condition. Other studies have also failed to find a clear relationship between lead load and body condition in wild birds (Roux and Marra 2007, Snoeijs et al. 2004), and a number of experimental studies involving the supplementing of high lead food to wild birds have likewise found only limited impact on growth and physiological condition (Ruuskanen et al. 2015, Eeva et al. 2014). There are several potential explanations for this lack of relationship in the current study. It may be that the degree of lead contamination occurring among mockingbirds in New Orleans may not have a measurable impact on physiological condition. Alternatively, it is possible that more sophisticated measures of condition (e.g., blood chemistry), or samples taken during more stressful conditions might detect differences (Roux and Marra 2007).

5. Conclusion

We report correlational evidence that lead levels in blood and inert tissue of an urban songbird accurately reflects local concentrations of lead in the soil, and that higher exposure to lead is associated with increased aggression toward conspecifics. These findings suggest that sub-lethal lead exposure can have potentially serious behavioral impacts on urban wildlife, similar to those observed in better documented human and laboratory animal systems. Given the sheer number of individuals of wildlife and pets that are likely to come into regular contact with lead-contaminated soil, our hope is that this study will stimulate further research into pathways of ingestion and short-term and long-term consequences of sub-lethal exposure to lead in urban animals.

Acknowledgements

We thank D. Henry, G. Londoño, J. Phillips, N. Frumkin, M. Schmidt, J. Ricou, E. Iverson, S. McCombs and members of the Karubian lab for assistance with logistics, fieldwork or song analyses, and for valuable feedback. D. Gammon and C. Botero provided invaluable advice on analyses of mockingbird songs. We thank the many residents of New Orleans who allowed us to work on their properties. Research was supported by a post-doctoral fellowship to RDR from the American Association of University Women and grants from the Morris Animal Foundation, Tulane University's Center for Bioenvironmental Research, the Center for Engaged Learning and Teaching, the Newcomb-Tulane College Dean's Office, and the Newcomb College Institute.

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