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Investigating Behavioral Responses to Lead (Pb) Exposure in a Cosmopolitan, Urban Songbird

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APPROVAL PAGE

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COMPLIANCE PAGE

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ABSTRACT

Globally, the contamination of anthropogenically-disturbed environments with lead (Pb) pollution constitutes a major threat for exposed organisms. While Pb is widely known to be toxic at high levels of exposure, recent literature has shown that sublethal levels of this toxin cause a suite of deleterious effects on individual organismal systems. However, there remain considerable holes in our understanding of how multi-systematic and sensitive responses like behavior are impacted by this form of pollution. Moreover, recent work has suggested that the high selective pressure caused by chronic Pb exposure may drive the resistance and local adaptation of terrestrial organisms. However, these blossoming lines of research lack evidence on whether behavioral responses may be subject to this pollutant-incurred adaptation.

To better understand the effects of sublethal Pb on organismal behavior, Chapter 1 utilizes an experimental design to examine the effect of sublethal Pb exposure on three key behaviors in an avian model-House Sparrows (*Passer domesticus*). Specifically, we examined escape flight performance, activity in a novel environment, and in-hand aggression; behaviors that have all been shown to be crucial for avian fitness. Compared to controls, birds exposed to environmentally-relevant levels of sublethal Pb exhibited significant decreases in metrics related to escape flight performance, as well as and movement activity. However, our in-hand aggression assessments (number of struggles, average breathing rate, agonism score) did not capture any Pb-induced behavioral shifts. Together, these results indicate that sublethal Pb exposure selectively impacts different behavioral endpoints with those reliant on more biomechanical attributes more susceptible to this pollutants' deleterious effects. A clearer understanding in how sublethal Pb impacts the behavior of commensal species like house sparrows provides a vantage point into how this toxin may impact other organisms inhabiting anthropogenically-polluted environments.

In Chapter 2 we investigate the scale of Pb-influenced selection on avian behaviors by sampling a population of House Sparrows in Broken Hill, an Australian mining town chronically-polluted with Pb. We caught 600+ birds across a well-characterized soil Pb gradient and then conducted field-modified versions of the same behavioral assays of escape flight performance, activity in a novel environment, and in-hand aggression. We found that the birds caught in sites of high Pb exposure exhibited impaired takeoff flight performance, as well as a decreased average breathing rate when compared to birds caught in low Pb-polluted sites. Conversely, there was no differences among high and low Pb birds' movement activity scores, as well as the other two in-hand aggression metrics of number of struggles and agonism score. Collectively, our results suggest that while local adaptation may be occurring for some behaviors, this adaptation is likely incomplete for other vital behavioral responses.

TABLE OF CONTENTS

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This Thesis is dedicated to my family, friends, and the continued pursuit of knowledge that bring us all together.

“Nothing is impossible-not if you can imagine it.
That's what being is a scientist is all about.”
-Futurama

LIST OF TABLES

LIST OF FIGURES

Chapter 1. Sublethal Pb Exposure Impacts Takeoff Flight Performance and Movement Activity but not Aggression in a Commensal Songbird

Introduction

Lead (Pb) is a widespread and toxic heavy metal pollutant that poses a major threat to humans and wildlife alike (Assi *et al.* 2016). While Pb is known to be naturally present in small quantities within the Earth's crust, anthropogenic extraction and use of the metal has greatly increased over the past two centuries (Marx *et al.* 2016). Globally, Pb is commonly used in the manufacture of many products such as batteries, water pipes, paints, firearm ammunition, cable sheaths, and, until recently, gasoline (Cheng and Hu 2010, Assi *et al.* 2016, Williams *et al.* 2017). The industrial manufacturing processes to make these products, as well as improper waste control, coal burning, water runoff, urbanization and the extraction of the metal itself (Cheng and Hu 2010, Cai and Calisi 2016, Mclennon-Gillings 2022) has polluted an increasing number environments across the world with Pb, exposing a growing number of organisms to this toxic stressor.

Once Pb enters organisms' bodies through exposure to contaminated water, soil, dust, air, or food (Cheng and Hu 2010, Levin *et al.* 2008), it adversely affects the function of organisms' internal processes (Assi *et al.* 2016, Neal and Guilarte

2010, Franson and Pain 2011). Continuous or heightened exposure to Pb causes the accumulation of this pollutant in organisms' tissues where it exacerbates these toxic effects, potentially leading to mortality (Franson and Pain 2011). While the lethality of Pb in organisms has been widely studied over the years, organisms are not typically exposed to Pb levels leading to immediate death (Pain *et al.* 2019). Instead they are exposed to sublethal levels of Pb which, while also known to incur deleterious effects on organisms, have received less comprehensive study.

Sublethal Pb exposure has been found to negatively impact a multitude of organismal systems that may contribute to decreased individual survival across a variety of taxa. As Pb itself often acts as a reactive oxygen species, many studies have found that sublethal Pb exposure increases oxidative stress biomarkers associated with decreases in the health of blood cells (Arriata *et al.* 2004, Chiesa *et al.* 2006, Koivula and Eeva 2010, Cid *et al.* 2018), digestive tissue (Radawan *et al.* 2010, Lee *et al.* 2019), reproductive organs (Rodríguez-Estival *et al.* 2016, Vallverdú-Coll *et al.* 2016), and more. In addition to decreasing cellular health in these areas, sublethal Pb exposure has also been found to decrease a plethora of parameters associated with healthy immune function in a variety of fish, birds, and mammals (Lee *et al.* 2019, Vallverdú-Coll *et al.* 2019, Redig *et al.* 1991, Miller *et al.* 1998). Sublethal Pb has also been known to adversely affect multiple species' skeletal systems through altering bone composition, or impairing bone formation and repair (Gangoso *et al.* 2009, Beier *et al.* 2013, Ishii *et al.* 2018).

Most prominently, Pb has repeatedly been found to decrease the health and function of many organisms' neural tissue or synaptic function (Mateo *et al.* 2003*,* Jin *et al.* 2019, Nixdorf *et al.* 1997, Zou *et al.* 2020), incurring downstream cognitive deficiencies (Monchanin et al. 2021, Burger and Gochfeld 2000, Ishaque et al. 2020, Levin et al. 2008). While there is substantial evidence linking sublethal Pb exposure to decreases in individual physiological measures, less research has focused on larger-scale responses that encompass multiple organismal systems. As such, a more comprehensive understanding of how sublethal Pb impacts multi-systematic, frontline responses like behavior is vital for further understanding how Pb pollution impacts organismal survival.

An organisms' behavior serves as a sensitive indicator that reflects on the condition and functioning of multiple internal systems. Since behaviors are at the forefront of where major environmental changes meets an organism's internal processes, shifts in behaviors are often the first places where the effects of environmental stressors can be observed (Peterson *et al.* 2017). This allows organisms' behavioral outcomes to serve as "early-warning" indicators of organismal health in response to changes in environmental quality (Hellou 2011). Furthermore, these early signs are often noticeable changes that can be observed and quantified even at sublethal levels of pollutant exposure (Whitney and Cristol 2018, Pain *et al.*2019, Peterson *et al.* 2017), highlighting the usefulness of behavior in picking up subtle or sensitive changes associated with environmental contamination. Lastly, as behavioral responses encompass multiple organismal systems, examining these responses allows for the assessment of not only the efficacy of individual systems, but also their ability to work in tandem to produce more complex behaviors (Scott and Sloman 2004). This not only provides an additional dimension for identifying potential impairments incurred by pollutants, but also allows for researchers to measure the health of multiple systems that may already be known to be adversely impacted by pollutants like Pb.

The flexibility and scope associated with behavioral responses has proven to be highly comprehensive and useful in assessing individual condition for wildlife living in Pb-polluted environments. Multiple studies have demonstrated how certain behaviors and behavioral states are affected by sublethal Pb contamination across different species of birds (McClelland *et al.* 2019, Grunst *et al.* 2018, Gorissen *et al.* 2005), fish (O'Neal 2021, Weber 1993), amphibians (Steele *et al.* 1989, Chen *et al.* 2006) and insects (Mogren and Trumble 2010, Migula and Binkowska 1993, Scott *et al.* 2022). While most of these studies have specifically examined how Pb-contaminated environments rather than Pb itself has affected organismal behavior, they lend support for the ability to use behavior as a comprehensive biomarker of Pb's affect on organismal function and well-being.

To better understand the effects of sublethal Pb on organismal behavior, we studied three key behaviors after exposure to an environmentally-relevant levels of Pb. Specifically, we chose to examine takeoff flight performance, activity in a novel environment, and in-hand aggression These behaviors have all been shown to be crucial for avian survival and fitness (Lima 1993, McGown *et al*. 2015, Kluen *et al.* 2014). Considering the well-known, often-systematic effects of sublethal Pb on avian physiological and neurological function (Cid *et al.* 2018, Redig *et al.* 1991, Mateo *et al*. 2003, Burger and Gochfeld 2000), we hypothesized that birds exposed Pb would suffer deficiencies in their biomechanical flight performance in the context of escape from a simulated predation event (Carlson et al 2014, Veasey et al. 2001). Due to the aforementioned physiological and neurological deficiencies incurred by Pb, we similarly surmised that birds exposed to sublethal Pb would show less overall locomotive activity within the context of a novel environment assay (Grunst *et al.* 2018). Finally, based on past research (McClelland *et al.* 2019, Janssens *et al.* 2003) as well as known Pb-incurred impairments on the avian neurological system, we hypothesized that Pb exposure would interfere with the behavioral response of being handled by a simulated predator.

We chose to use the House Sparrow (*Passer* domesticus) as an avian model to assess the potential effects of sublethal Pb on these behaviors. House sparrows are sedentary, non-migratory, and globally-distributed songbirds that are intimately associated with human-dominated landscapes (Havlíček *et al.* 2022, Kekonnen *et al.* 2011, Swaileh and Sansur 2006); the very environments most likely to experience sublethal Pb pollution (Roux and Marra 2007). This close association between house sparrows and human development has made them a good representative for how heavy metal pollution may be affecting other birds and organisms in the surrounding area (Swaileh and Sansur 2006, Gragnaniello *et al.* 2001). The combination of a cosmopolitan distribution, relative abundance, sedentary ecology, and a highly commensal relationship with humans have made house sparrows a desirable model species for assessing the effects of sublethal Pb on behavior and in doing so, contributing to a greater understanding of how this stressor may impact organismal survival.

Methods

House sparrow acquisition and care

We captured (mist-nets) house sparrows at five sites between Newport News, Williamsburg, and York counties in Virginia, USA (37.119382, - 76.465386) between February-March (pilot studies 1 and 2, n = 24) and September-November (main study, n = 55) of 2022 (Fig. 1). Once caught, birds were immediately transferred to a bird bag or holding bucket and transported to the nearby College of William & Mary aviary in Williamsburg, VA.

Upon capture, birds were housed in outdoor aviaries (approximately 4 x 3 x 2.5 m) in groups of 12-18 birds per cage with ad libitum access to food (50% Healthy Harvest “chicken starter crumbs”, 50% Volkman “finch super seed blend”), oyster shell grit, and drinking water. We banded each bird with unique combinations of colored plastic hobby beads (Perler) on their tarsi so we could identify individuals. During December 2022, immediately before the start of the main study, we installed a heat lamp (Flukers’s Reptia-Clamp Lamp 8.5in with 250w, heat-emitting bulb) in each aviary to help the birds stay warm.

Pilot study 1

To inform the design of the main study, and generate exposure levels of Pb reflective of other cases of Pb contamination, we conducted a 12-week pilot study. We arbitrarily assigned 20 house sparrows (9 female, 11 male) into 5 groups (n = 4 in each) ensuring an even sex ratio in all but one group. We placed each group in a separate aviary with the housing conditions described above.

To expose birds to Pb we weighed crystals of Pb acetate ($Pb(C_2H_3O_2)_2$) and then dissolved them into DI water. We placed two water dispensers in each aviary. Each dispenser contained 150 mLs of the appropriate treatment solution. These dispensers were the only source of water available for the birds. Water dispensers had one small trough at the base so that birds could drink the water with minimal splashing/bathing in the dispenser. Once exposure began, we refreshed the treatment water every 2 days so that the birds had continuous access to the appropriate Pb-exposed water for the duration of the experiment.

We assigned each group to one of five treatments in which birds were exposed to lead in their drinking water from March-July, 2022. The five pilot treatments included a control (no Pb added to tap water), treatment 1 (Pb/DI water concentration of 100ug/dL), treatment 2 (Pb/DI water concentration of 200ug/dL), treatment 3 (Pb/DI water concentration of 400ug/dL), treatment 4 (Pb/DI water concentration of 800ug/dL). We chose these concentrations of Pb with the intention of reaching ecologically relevant levels of Pb in the blood of the birds, using the conversion calculations reported in Goodchild *et al.* (2021). For the four Pb-exposure treatments, in ascending order, the intended target blood-lead concentrations were: 5 ug/dL (unpublished data from house sparrows caught in

Flint, MI, USA, Dorothy Zahor), 10 ug/dL (Chandler *et al.* 2004, Zahor *et al.* 2021), 20 ug/dL (Franson and Pain 2011), and 40 ug/dL (Harris 2022).

To measure the amount of Pb in the birds' blood over time, we collected a blood sample from half of the birds in each treatment aviary once per week. In other words, each bird was bled once every two weeks and two (of the four) birds from each treatment were sampled every week. To collect blood we carefully punctured the brachial vein with a surgical needle and drew the emerging droplet of blood into a capillary tube. We immediately analyzed each blood sample using a LeadCare II system (Meridian Bioscience) to generate a measurement of blood-lead concentration (ug/dL).

Due to lack of timely availability of LeadCare assay kits from the manufacturer during the first 6 weeks of this pilot study, we stored collected blood at -80°C in EDTA Eppendorf tubes, later defrosted these samples at room temperature, and then immediately analyzed them as described above. To ensure a sufficient quality of Pb measurement, we performed the LeadCare systems' quality control procedure on a monthly basis, or upon the opening of a new lot of assay kits. This involved mixing a provided solution of dissolved Pb into an assay kit and ensuring the resulting Pb concentration readout was within a set range. Following 12 weeks of exposure to Pb and blood sampling, we ethically euthanized all the Pb-exposed birds. We retained the four control birds for a second, much smaller, pilot study.

Pilot study 2

The Pb concentrations in the blood of house sparrows in pilot study 1 were unexpectantly low (see Results), hence we decided to perform a small-scale, second pilot study employing higher concentrations of Pb in drinking water. We arbitrarily assigned two birds to receive Pb/DI water concentration of 1,600ug/dL (Treatment 5) and the other two birds to receive 3,200ug/dL (Treatment 6) in their water, keeping the treatments in separate aviaries with the same general housing conditions described above. Pilot study 2 ran over five weeks from 7 October- 9 November, during which we collected weekly blood samples from all birds (as described above) and analyzed them on the LeadCare II instrument to estimate blood-Pb levels. At the end of this experiment we humanely euthanized the four birds.

The intention with these two treatments was to create blood-Pb concentrations that were representative of highly contaminated environments (Harris 2022) while still being sublethal.

Comparison of LeadCare II and ICP-MS analysis of blood-Pb concentrations

The LeadCare II instrument determines blood-Pb concentrations via anodic stripping voltammetry and has been used in several wildlife studies because of its ease of use, portability, and relatively low cost (Herring et al. 2018, Zahor *et al* 2021, White *et al.* 2022, Harris 2022). The system also provides relatively quick results which was vital in allowing us to keep track of Pb levels on a weekly basis, and without needing to ship samples out for external analysis. However, the system was designed to analyze Pb concentration in human blood and is known to underreport blood-Pb concentrations of birds (Zahor *et al.* 2021, Harris 2022). To account for this, we compared LeadCare results to those generated by inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS offers a more-precise method that, while more time-intensive and often less practical, has shown to be highly correlated to other blood heavy metal analysis methods like graphite-furnace atomic absorption spectrometry (GFAAS) (Herring et al., 2018).

To generate data for this comparison, we collected blood samples from each bird in the main study (see below) after completion of all trials. Using blood collected from the same birds on the same day, we obtained a LeadCare measurement (as described above) and sent an additional blood sample to an external lab for ICP-MS analysis (Dartmouth TEA Lab, Hanover, NH, USA).We found a strong positive association between the LeadCare II and ICP-MS data (linear regression, $r^2 = 0.944$, $y = 1.57(x) + 1.294$). Using the equation from the linear regression, we were able to convert all LeadCare II estimates of blood-Pb to a value predicted by ICP-MS. Thus, all blood lead measurements subsequently reported from here on are of blood-Pb concentrations that have been adjusted to reflect this conversion.

Main study

After comparing the blood-Pb concentrations from birds in both pilot studies we selected two Pb-exposure concentrations for the main exposure experiment: a “lower” treatment of 800 ug/dL (Treatment 4) and a “higher” treatment of 1,600 ug/dL (Treatment 5). Based on the pilot studies, we predicted that the “lower” treatment would result in a blood-Pb concentration of approximately 14 ug/dL, which has been reported in birds occupying polluted areas of Flint, MI, USA (Zahor *et al.* 2021). We predicted the “higher” treatment would result in blood-Pb concentrations of approximately 20 ug/dL which is likely near the limit of sub-lethal exposure for wild birds (Franson and Pain 2011).

We conducted the main study between December 2022-March 2023. We arbitrarily assigned 54 wild-caught house sparrows (18 female, 36 male) to three treatment groups (control, lower, higher, n = 18 in each treatment)) where we maintained this 1:2 sex ratio in each group. Each treatment group was divided into three aviaries (n = 6 per aviary, 2 female, 4 male) identical to the general housing described previously. Based on how blood-Pb concentrations increased and reached an asymptote over time in the pilot study 1 (see Results, Fig. 2a) we decided to expose birds to Pb for approximately 9 weeks.

After 9 weeks of exposure, each bird experienced two behavioral assays, escape takeoff flight, and in-hand aggression (described below), within 24 hours of each other. The day after completion of these behavioral assays, we collected blood samples (as described earlier) for LeadCare II analysis. Following a further 5-6 days of continued exposure to Pb, each bird experienced a third behavioral assay, an activity assay (described below). Three weeks after the completion of the activity assay, we collected terminal blood samples to compare blood-Pb concentrations analyzed by LeadCare II and ICP-Ms, as described above.

Two birds (1 control, 1 higher treatment) died from unknown causes before they performed behavioral assays. A further bird from the higher treatment experienced a leg injury in the earliest parts of the study and was excluded from escape takeoff and activity assays. Hence the sample size was reduced in this treatment group.

Escape takeoff flight assay

We conducted escape takeoff flight assays from 11:00-15:00 in a long flight aviary (approximately 12 x 9 x 2.5m) that was adjacent to but visually separated from the birds' housing aviaries. Within the flight aviary, we built a "flight studio" structure to encourage the birds to fly in a similar direction upon release (Fig. 3). The flight studio consisted of two upright wooden boards (180 x 120 cm) on the sides and an upright but shorter wooden barrier (120 x 80cm) at the end towards which the bird was encouraged to fly toward. At the opposite end to the barrier, we fixed a 4 cm long wooden dowel to a wooden beam that was at ground level. The perch was the release point for each bird. We also hung a heavy drop-cloth from the right side of the studio to further incentivize the bird to fly through the flight studio and not around it. With the experimenter stationed on the left side of the studio to release the bird, this whole arrangement encouraged birds to fly from the release perch directly toward and over the end-barrier.

Before a series of flight trials, all birds in an aviary were caught and weighed to 0.1 g precision (Denver Instrument Co., Model TL-203). Quickly after, the cameras were started and the flight trial initiated. Each bird was placed in a loose “banders grip” in which the bird was placed with its back on the palm of the observer’s hand, held with its neck between the observer’s index and middle finger. This grip was rotated downwards as to ensure the bird was grasping the release perch with its talons. When the bird was gripping the release perch and in a consistent body position, the experimenter removed their hand from the bird and the bird took off from the perch toward the end-barrier (height = 0.8m) of the flight studio. Once a bird exited the flight studio, they would be flushed by the experimenter towards perches placed on the far end of the flight aviary where they would be caught in a fine mist-net (70 denier, 2 ply) that was 2.5 m beyond the end-barrier of the flight studio. This enabled safe and quick bird recaptures. In total, 51 birds (control, n=17; lower, n=18; higher, n=16) completed escape takeoff flight trials.

To record each takeoff flight, we placed three GoPro Hero7 cameras (60 frames per second,1440 resolution) on a fixed PVC structure behind the release perch. The video cameras were synchronized (by ambient sound and flashlight cues) and activated before each series of flight trials. We then took additional measurements to calibrate the air space of the flight studio. We did this by slowly moving a 46 cm wand with two brightly colored polystyrene orbs on each end through the whole flight area. By digitizing the positions of the orbs in Argus (see below) we generated paired points that are a known distance from each other throughout the whole air space of the flight studio. We also recorded on each camera an L-shaped PVC structure that was placed in the center of the studio to serve as a reference to determine the spatial orientation of the x (left-right), y (close-far), and z (vertical up-down) axes. The wand was removed before we flew birds in the studio, but the L-shaped structure, placed unobtrusively on the ground, remained.

We defined the start (frame 1) of a takeoff flight as the first frame where a bird’s feet left the perch as it became airborne. In Argus (see below), we digitized the approximate centroid of the bird for the first 15 frames of flight. This period accounted for the section of flight where a bird is most likely sensitive to escape from a predator (Carlson *et al.* 2014, Swaddle *et al.* 1999).

Escape takeoff flight video analysis

We analyzed the video recordings of each flight from each camera in Python version 3.8.3 (Van Rossum and Drake 2009) using the open-source software package, Argus. We employed a previously-established protocol (Jackson *et al.* 2016, Meehan 2022, Swaddle *et al.* 2023) in which we reconstructed the three-dimensional flight path of each bird. Briefly, the Argus software aligned the frames from each camera using the sound and light cues that were recorded on each GoPro. It also accounts for the orientation of x, y, and z axes and uses the paired points generated by digitization of the orbs at either end of the calibration wand to assign real measurements to the volume of air space recorded on the cameras. Following manual digitization of the position of the centroid of each bird on each of the 15 frames of recordings, the software recreates the 3-D position in a calibrated air space so that we can generate metrics of flight performance. For more detail about this methodology see Jackson *et al.* 2016.

From the 3-D coordinates generated in Argus, we computed the energy, in Joules, expended during escape take-off (Swaddle et al. 1999). Specifically, we calculated instantaneous velocity by subtracting the value of the previous frame's (*n-1*) coordinates from the current (*n*). We multiplied the resultant vector magnitude by 60 (i.e. frame rate of recordings) to yield velocity (*v*) in m/s (Equation 1; Meehan 2022).

<u>Equation 1.</u>

$$v = \sqrt{(x_n - x_{n-1})^2 + (y - y_{n-1})^2 + (z_n - z_{n-1})^2 * 60}$$

We used the resulting instantaneous velocity along with the mass of each bird (*m*), the vertical height achieved in each frame (*z*), and the gravity constant (*g*) to calculate both the instantaneous kinetic and potential energy. These were summed to compute the total energy (*TE*) expended in each frame (Equation 2). We averaged the energy expended values over the 15 frames of takeoff flight to generate an average energy expenditure over escape takeoff flight.

<u>Equation 2.</u>

$$TE = \frac{1}{2}[(m * v^2)] + [(m * z * g)]$$

We also calculated the initial takeoff leap force (*TOF*) to quantify the force generated by the bird's legs over the first two frames of takeoff (Provini *et al.* 2012, Bosner and Rayner 1996). This was calculated by multiplying the acceleration of the bird in that second frame (sfv^2, in m/s^2) by each birds' mass (*m,* in kg) (Equation 3).

<u>Equation 3.</u>

$$TOF = sfv^2 * m$$

In-hand aggression assays

We conducted in-hand aggression assays from 10:00 to 16:00 in the long flight aviary. Birds (n = 52) were chosen at random independent of treatment and caught by an experimenter with a hand net in their housing cage. Once caught, the bird was placed in a bird bag for 2 mins to reduce catching stress and brought to another experimenter for testing. Using methods adapted from Brommer and Kluen (2012), a single experimenter assessed aggressive responses using three assays in the following order:

1) *Struggle assay*: The bird was taken from the bird bag and placed in banders' grip. The bird was then held vertically approximately 30cm from the experimenter's face. A stopwatch was started immediately upon being exposed to the experimenters' face and the number of struggles (defined as major movements of the body and/or head directed towards repositioning as would be needed in escaping from handling) were recorded for a 30s time period. A higher score represented a bird that struggled more as to try to fight or escape from the predator, and thus, was said exhibit an increasedaggressive response (Brommer and Kluen 2012).

2) *Breathing assay*: Immediately following the struggle assay, the bird was lowered to a horizontal position slightly closer to the experimenter's face (approximately 15cm) with it's legs fully secured by the experimenters' second hand as to expose the bird's chest. This allowed the experimenter to observe movements of the chest associated with breathing (Carere and van Oers 2004). The experimenter counted these movements for 30 s, to

estimate a breathing rate. Immediately following the first 30 s bout, the assay was repeated to generate a second estimate of breathing rate. We averaged these two estimates to generate a single breathing rate for each individual bird.

3) *Agonism score*: After these assays, the bird was assigned a subjective agonism score to reflect the overall aggression the individual displayed during this series of assays, as well as inter-assay handling/processing. This assessment takes into account any escape movements, biting, vocalizations, and pecking that may indicate a stronger response to avoiding handing. To quantify this, the score employs a scale that ranges from 1 (completely passive) to 5 (struggling during the entire time). A bird with a higher score was interpreted as being more aggressive (see "docility" in Brommer and Kluen 2012, Kluen *et al.* 2014).

Activity assay

We quantified metrics of locomotor activity of each bird when it was placed in novel test environment (Renner 1990) (Fig. 4). Similar assay-setups have been used to infer exploration (Dingemase *et al.* 2002, McGown *et al.* 2015, Huang *et al.* 2016, Moyers *et al.* 2018), risk-taking (Pobleté *et al.* 2021), and boldness (Quesada *et al.* 2022, McGown *et al.* 2015) of birds. We performed each activity assay between 09:00 and 15:00 in a soundproof, indoor aviary (3 × 3 × 2.5m).

Though birds were tested individually, we tested all individuals from the same treatment aviary sequentially. The order of treatment aviaries was randomized.

The activity arena comprised two sections: a small, dark release chamber (20 x 15 x 15cm) and a larger activity chamber (120 x 70.5 x 85cm) (Fig. 4). These two chambers were aligned so that when a door was opened, a focal bird could move from the release chamber to the activity chamber. The activity arena was constructed with plywood walls and floor and a translucent ceiling (Ejoy, 48L x 24W x 0.31in. thickness, corrugated poly-plastic roofing panel) that allowed the ambient light of the room to illuminate the activity chamber. We placed 4 perching structures (each with a single vertical post 69 cm tall and 3 horizontal perches that were 35 cm long, each constructed from 1.5 cm diameter bamboo) in the chamber. In addition, we attached an additional wooden-dowel perch (36cm long, 2 cm diameter) to the walls of the activity chamber as indicated in Figire 4. We also attached a GoPro (Hero7, 60fps, 1440 resolution) to the wall closest to the entrance of the activity chamber. Though we placed hardware wire deterrent above the camera, some birds tried to perch in that blind spot (13 attempts over 8 individuals); hence we counted the camera location as another perch. In total, there were 16 separate perch location (12 attached to free-standing vertical posts, 3 attached to the chamber walls, and then the GoPro camera). We marked the floor of the activity chamber with a grid pattern (rectangles, 30 x 18cm) so we could note which part of the chamber a bird occupied. Even during potential instances of the bird landing on the camera, birds could be visualized in all discrete perches and areas.

To begin an activity trial we placed a focal bird into the release chamber for 5 mins (Fig. 4) (McCown *et al.* 2015). Following the 5-minute period, we opened the door to the activity chamber. In most cases (n = 40 of 51) the bird entered the activity chamber immediately. In the 11 cases when this did not happen, the experimenter waited 10 s and tapped on the outside of the release chamber to encourage the bird to enter the activity chamber. All of these birds entered the activity chamber with that additional stimulation. We video recorded all the movements and behaviors of the focal bird for 8 mins. Following each activity trial, the focal bird was returned to its treatment aviary. We cleaned the activity chamber if there were droppings or loose feathers so as to minimize cues left from the previous trial.

We analyzed the 8-minute activity videos (n = 51) to quantify five behaviors:

1. Number of “flights”, defined as the total number of a movements of the focal bird that involved use of its the wings, and/or movements between perches.
2. Number of “hops”, defined as the total number of leg-based bouts of movement on the floor of the chamber or along a single perch
3. Number of different “perches visited”, defined as the total number of unique perches (max = 16) that a bird visited
4. Number of different “areas visited”, defined as the total number of unique grid zones (max = 16) the bird visited.

5. Number of “self-maintenance behaviors”, defined as the total number of bouts of self-maintenance activity, including preening and bill wiping.

Statistical Analyses

To assess the effect of Pb exposure on escape takeoff flight, we compared average energy expended during takeoff across the 3 treatments (control, lower, higher) with a one-way ANOVA. After running our ANOVA, used a Tukey’s post-hoc test to examine differences between our individual treatments. To ensure our results met the normality assumption for using an ANOVA, we conducted a Shapiro-Wilks test on extracted residuals. The same statistical process was repeated for the other flight metric, takeoff force.

Similarly, we examined among-treatment differences in the breathing assay metric with a one-way ANOVA and outcomes from the struggle assay and agonism score with separate Kruskal-Wallis rank sum tests.

We reduced the dimensionality of the five behavioral metrics of the activity assay using principal components analysis (PCA). The PCA rendered two interpretable principle components (see Results for descriptions). We compared among-treatment differences in PC1 and PC2 using separate Kruskal-Wallis rank sum tests. If significant, we performed the accompanying post-hoc test, the Dunn test, to tease apart differences between treatments.

All analyses were performed in R version 4.2.1 (R Development Core Team, 2021) interpreting two-tailed tests of probability.

Results

Pilot 1 and Pilot 2

Sparrow Pb levels during our first pilot (Fig. 2a) increased quickly during the first 4 weeks of exposure before decreasing slightly over the next 8 weeks. During these next 8 weeks, the Pb levels reached a relative asymptote. At the end of our study, the adjusted average Pb levels in each cage were: 0ug/dL for our control, 0ug/dL for treatment 1, 6.279ug/dL for treatment 2, 9.183 for treatment 3, and 17.229ug/dL for treatment 4.

In our second pilot, we observed a similar trend with both treatments experiencing a rapid elevation of blood Pb levels during the first 3 weeks of exposure (Fig. 2b). After 4 weeks, we noted a general levelling-off of Pb levels and after taking a final measurement (treatment 5 average = 33.715ug/dL; treatment 6 average = 48.159ug/dL), ended the study earlier than the previous pilot.

Main study

The blood samples taken closest to all three behavioral assays showed that birds in the lower treatment group had an average (± standard error) blood Pb concentration of 16.678 (± 3.931) ug/dL. Birds in the higher treatment had an average of 30.041 (± 7.081) ug/dL. Two of the 18 control birds had detectable Pb in their blood (6.643ug/dL and 6.801ug/dL). All other control birds consistently had Pb levels that were below the detectable level of our LeadCare II system (<6.48 ug/dL).

Escape takeoff flight assay

Exposure to Pb influenced energy expended during takeoff flight (Fig. 5a; ANOVA, $F_2 = 9.711$, $p = 0.0003$). In particular, birds in both the lower (Tukey HSD, $q = -0.034$, $p = 0.007$), and higher (Tukey HSD, $q = -0.0469$, $p = 0.0003$). Pb treatments expended less energy than controls. However, we did not detect differences between the lower treatment and higher treatments (Tukey HSD, $q = -0.013$, $p = 0.487$). Similarly, Pb influenced takeoff leap force (Fig. 5b; ANOVA, $F_2 = 33.64$, $p < 0.000001$) such that birds in both lower (Tukey HSD, $q = -1.901$, $p < 0.0001$) and higher (Tukey HSD, $q = -2.336$, $p < 0.0001$) Pb treatments exerted less force in their takeoff leap than controls There was no difference in takeoff leap force between birds in lower and higher Pb treatments (Tukey HSD, $q = -0.435$, $p = 0.109$).

In-hand aggression assays

Exposure to Pb did not influence the three in-hand metrics of aggression in terms of number of struggles, average breathing rate, or agonism score (Table 1).

Activity assay

The PCA of the five activity metrics returned two PCs, which explained 87.5% of the original variation (Table 2. PC1,which we interpreted as total “movement”, explained 69% of the variation and was positively loaded with all 5 behaviors. PC2, which we interpreted as “sedentary self-maintenance”, explained an additional 18.5% of the variation and was positively loaded with “areas visited” and “hops” and negatively loaded with “self-maintenance” (Fig. 6a).

Exposure to Pb influenced PC1 (movement) scores (Kruskal-Wallis, $x^2 = 12.6$, df = 2, p = 0.0018) but not PC2 (sedentary self-maintenance) scores of birds (Fig. 6b; Kruskal-Wallis, $x^2 = 0.120$, df = 2, p = 0.942). Within the PC1 results, birds from both lower (Dunn, z = 3.444, p = 0.0009) and higher (Dunn, z = 2.467 , p = 0.021) Pb treatments had lower PC1 (movement) scores than controls. There was no difference in PC1 scores between birds in lower and higher Pb treatments (Dunn, z = 0.889 , p = 0.561).

Discussion

House sparrows exposed to sublethal levels of Pb exhibited decreased performance in our biomechanically-centered assays of takeoff flight and movement activity. In takeoff, birds that were exposed to Pb exhibited decreased performance in both the initial leap from the perch and their flight as they left the ground. In the movement assay, birds that had been exposed to Pb were less active. While both of our Pb treatments differed from controls, we did not see differences between our lower and higher Pb treatment groups. This is consistent with the lower Pb treatment being above a threshold that induces decreases in biomechanical performance.

It is likely that the Pb-induced performance reductions we observed in both of these biomechanical and movement assays would have negative implications for avian survival in nature. Less energetic takeoff from the ground would likely increase the risk of predation (Lind *et al.* 2010), When compared to our controls, birds in our lower Pb treatment group exhibited a 31% decrease in average energy expended across the start of takeoff flight. Similarly, birds in our higher Pb treatment group exhibited a 24% decrease in energy during the start of their flight. The metric we used is a summation of kinetic and potential energy (Williams and Swaddle 2003, Swaddle *et al.* 1999), hence birds expending less total energy during their takeoff flight were generally moving more slowly and gaining less height from the ground over time. In takeoff flight, where the majority of biomechanical forces are generated by the pectoralis muscles of birds, we similarly observed Pb-induced decreases in takeoff leap force as the birds left the perch. The power generated for this leap is largely generated by hindlimb muscles, representing another internal region crucial for flight physiological performance (Henry *et al.* 2005). In particular, control birds utilized 45% and 55% more force in their takeoff leap compared to birds in the lower and higher Pb treatment groups, respectively. Interpreting the leap and takeoff flight together, it is clear that exposure to sublethal Pb leads to substantially decreased biomechanical performance of several systems in the avian body.

The Pb-induced reductions in takeoff performance likely influence the ability to escape from a predator (Fernández-Juric *et al.* 2006). In the case of a songbird evading a ground-based predator, the start of takeoff from the ground is the most crucial part of flight (Veasey *et al.* 2001, Jones *et al.* 2009). Therefore, individuals moving more slowly and gaining less height are less likely to evade predation (Witter *et al.* 1994, Swaddle *et al.* 1999). Apart from anti-predator evasion, decreased flight performance may have detrimental consequences for other avian activities such as seasonal migration (Seewagen 2020, Perez *et al.* 2007), food provisioning (Crino *et al.* 2017), reproductive output (Byers *et al.* 2010), and resource competition (Altshuler 2006, Ecke *et al.* 2017). Therefore, Pb-induced decreases in flight performance may result in a suite of outcomes that likely lower avian fitness.

Consistent with decreases in takeoff performance, overall movement activity was reduced in birds exposed to sublethal levels of Pb. Our data revealed that birds in the lower treatment group experienced an average 69% decrease across the five behaviors we examined (total numbers of flights, hops, areas visited, perches visited, and self-maintenance behaviors). Meanwhile, birds in the higher treatment groups experienced an average decrease of 52% among these behaviors. These activity decreases were also exhibited in our analysis of the movement activity score (PC1).

Activities associated with movement are associated with many aspects of avian behavior. Hence, decreases in overall movement activity due to Pb exposure may result in a variety of context-dependent effects. Birds not actively moving around their environment, particularly a novel one, may be less likely to find conspecifics, avoid predators, find shelter, and exploit resources such as suitable food (Huang *et al.* 2012, Bonter *et al.* 2013, Dominoni *et al.* 2020, Greenberg 1990). Furthermore, a lack of spatial movement could also reduce a bird's ability to gather information on their local environment. Even considering many birds' wide field of view, movement is commonly necessary to properly sense as well as gain information on their surroundings (Fernandez-Juricic 2012, Greenberg and Mettke-Hofmann 2001). While there are some indirect metabolic and physiological costs associated with engaging in movement-intensive behaviors (Careau *et al.* 2008, Owen-Ashley *et al.* 2006), larger opportunity costs associated with not exploiting resources and perceiving changes in their environment can be major drivers of survival for birds.

The Pb-affected mechanisms that cause the decreases in biomechanical and movement performance are unknown at this stage. This could include a suite of physiological and neurological mechanisms that are known to influence the behaviors we studied (Swaddle and Witter 1997, Provini *et al.* 2012, Careau *et al.* 2008). Exposure to Pb likely influences these types of mechanisms. For example, multiple measures of avian physiology are negatively impacted by Pb, such as inhibition of δ-aminolaevulinic acid dehydratase (ALAD), an enzyme involved with the heme biosynthetic pathway (Beyer *et al.* 2004, Cid *et al.* 2018, Sato *et al.* 2022, Holladay *et al.* 2012, Finley *et al.* 1976). Other indicators of hematological health can also be affected by exposure to Pb, such as packed cell volume (Beyer *et al.* 1988, Kerr *et al.* 2011), plasma protein concentration (Kerr *et al.* 2011, Vallverdú-Col *et al.* 2011), the H/L ratio (Bauerová *et al.* 2017), and hemoglobin levels (Beyer *et al.* 1988). Proper hematological responses play a crucial role in birds' abilities to regulate oxygen around their body (Butler 2016); hence, impairment in hematological responses may compromise oxygen-intensive behaviors such as takeoff and other movements. It is also possible that fundamental properties of the avian skeleton could be compromised by exposure to Pb, as the heavy metal competes with calcium during bone strengthening and repair (Mateo *et al.* 2003, Ishii *et al.* 2018). Given how important bone structure is for birds' oxygen regulation and flight ability (Butler 2016), increased Pb uptake by skeletal systems could make biomechanical movements and powered flight less efficient.

In addition to physiological impairments, exposure to Pb may also interfere with appropriate neurological functions. The brain and other parts of the central nervous system are targets of Pb accumulation in a several bird species (Douglas-Stroebel *et al.* 2005, Nam *et al.* 2012). In addition, Pb is a competitor of calcium at neural synapses and affects pre-synaptic and post-synaptic neurological functioning in the vertebrate brain (Neil and Guilarte 2010). In birds, Pb exposure has been associated with decreases in GABA and glutamate-related aspects of neurotransmission, as these biomarkers are most sensitive to interacting with Pb's oxidative properties (De la Casa-Resino *et al.* 2015, Nam *et al.* 2012, Cid *et al* . 2018). At a more structural level, exposure to Pb has been associated with increased inflammatory responses, histopathological damage, demyelization, and apoptosis of neurons in vertebrates (Zheng *et al.* 2022, Hossain *et al.* 2016). All of these are non-mutually exclusive mechanisms for how exposure to Pb may result in the decreased biomechanical and motor performance we observed in house sparrows.

We did not find any reliable evidence that exposure to Pb influences the three metrics associated with in-hand responses to an experimenter. This could mean that the birds' assessments of risk is little affected by Pb but it is more likely that the prior handling and longer-term captive housing of the house sparrows made them generally less reactive to these handling-based assays. These assays were originally developed and implemented in a study with wild-caught birds that were not acclimated to handling or captive housing. We posit that the repeated exposure to people (e.g. daily husbandry and animal care) rendered all birds more acclimated to human presence, making these assays less sensitive to assessment of handling stress and aggression than we had originally thought. There is precedent that exposure to Pb influences aggressive behavior (McClelland *et al.* 2019, Janssens *et al.* 2003), but we think our form of in-hand assay was not sufficient to detect similar effects.

The induced concentrations of Pb we observed in the house sparrow's blood from our lower treatment (average = 16.67 µg/dL) are reasonably reflective of those found in wild birds in polluted environments. For example, American robins (*Turdus migratorius)* living in urban areas contaminated by water runoff in Flint, MI, USA during the ongoing Flint Water Crisis were found to exhibit an average of 14.1 and a maximum of 32.0 ug/dL of Pb in their blood (Zahor *et al.* 2021). In addition, several populations of great tits (*Parus major*) living in polluted areas in Europe displayed blood-Pb concentrations within the range of about 10-28 ug/dL (Geens *et al.* 2010, Bauerová *et al.* 2020), as did mallards (*Anas platyrhynchos*) in polluted areas in Poland (Binkowski and Meissner 2013).

There is some precedent for the blood-Pb concentrations observed in our higher treatment (average = 30.04 µg/dL) to be found in free-living bird populations. For example, pied flycatchers (*Ficedula hypoleuca*) that lived close to an ore smelter in Finland reported blood-Pb concentrations of up to 42 μg/dL (Berglund *et al.* 2010). Similarly, resident house sparrows living close to an ore smelting facility in Broken Hill, Australia, were observed to have an average blood-Pb concentration of 39.8 ug/dL (Harris 2022). While relatively uncommon, we posit that our higher treatment level is useful for interpreting the effects of more extreme contamination events in nature. It seems likely that birds exposed to this concentration of Pb experience “subclinical poisoning”, which refers to a level of Pb exposure where physiological effects are reported despite not obviously influencing mortality (Franson and Pain 2010).

The negative impact of Pb exposure on birds' behavior, specifically their takeoff and escape flight performance, has significant ecological consequences that deserve careful consideration. Firstly, the observed decreases in takeoff flight performance indicate that Pb-exposed birds are more susceptible to depredation. While this has implications for individual fitness, it also may lead to a more-frequent pathway for Pb to bioaccumulate in predatory species through the food chain, leading to biomagnification and subsequent damage to these higher-trophic level organisms (Eindower *et al.* 2018; Abbasi *et al.* 2015). However, the ecological effects of Pb exposure are not limited to upper trophic levels alone. The observed decreases in both takeoff flight performance and general activity levels suggest that birds under the influence of sublethal Pb would struggle with various aspects of their existence that rely on these biomechanical processes.

Consequently, these birds are more likely to face non-predatory endpoints such as food scarcity, illness, or an inability to migrate, resulting in increased mortality rates. The bodies of these Pb-contaminated birds, upon death, may then be consumed by scavengers and decomposers, potentially introducing Pb to other species and the wider ecological community. Furthermore, the mobility and migratory nature of birds amplify the potential spread of Pb contamination. Birds inhabit vast areas and undertake migratory journeys that encompass multiple locations (Parolini *et al.,*2021). Since trace metals have been found in birds' organs and feathers along their entire migratory routes (Provencher *et al.,* 2016. Parolini *et al.* 2021), heavily contaminated birds may move to previously uncontaminated areas. However, due to their compromised behavioral functions, these birds are more vulnerable to predation or other risks in their new habitats. Consequently, an increased number of predators and organisms may be exposed to sublethal Pb levels and the associated detrimental effects. Considering these factors, the large-scale ecological consequences of Pb exposure on avian behavior extend beyond individual birds, potentially affecting the entire ecological community through bioaccumulation, biomagnification, and the spread of contamination via avian movement patterns.

The individual fitness deficiencies incurred through Pb’s deleterious effects on avian behavior may suggest long-term ramifications for the evolutionary trajectory of birds in Pb-polluted areas. The behavioral issues caused by Pb would likely constitute a major stressor for birds that, over time, would cause much higher selection for individuals with genetic traits that confer greater tolerance or resistance Pb toxicity (Klerks and Weis 1987). Over time, these selected birds would possess physiological and/or biochemical adaptations that enable them to better cope with the detrimental effects of Pb exposure. Consequently, these adaptations would enhance the survival and reproductive success of individuals in contaminated environments, leading to a gradual shift in the population towards increased resistance to Pb. However, it is crucial to recognize that selection for specific traits related to Pb tolerance may come at a cost. For instance, investing resources in detoxification mechanisms or repair processes may divert energy and resources away from other fitness-related traits, such as reproductive output or immune function. Therefore, the evolutionary response to Pb contamination might involve trade-offs, where birds with heightened Pb tolerance may exhibit reduced fitness in other aspects of their lives (Fritch *et al.* 2019). Observationally, this process may be already underway in isolated house sparrow populations within Pb-polluted mining towns. A recent study found that the expression of genes associated with cellular metal ion transport were more abundant in populations of house sparrows in these polluted mining towns, suggesting that these populations were at some intermediary state towards adaptation to this metal pollution found across the towns (Andrew *et al.* 2019). Although additional research is needed to make accurate predictions about the adaptive trajectories of these particular populations (see Ch. 2), the existing knowledge about the detrimental impacts of Pb, coupled with the observed adaptations in these potentially evolving populations, offers valuable insights into the mechanisms through which birds can potentially withstand and evolve in response to the negative effects of this stressor.

Lastly, the selection of the highly-commensal House Sparrow for this study has important implications for understanding the potential effects of Pb on other urban animals, as well as for human health. Pb pollution is typically associated with anthropogenically-disturbed environments, and House Sparrows, known for their preference for such habitats, have proven to be effective models to study of Pb pollution in these areas (Swaileh and Sansur 2006, Kekkonen et al. 2012, White et al. 2022, Gillings-Mclennon 2022). Already being a bioindicator of ambient Pb levels, there is high potential for this species to serve as a sentinel for assessing the impact of high Pb levels on the health of other organisms in contaminated areas, particularly other birds. Unlike many other bird species, House Sparrows are sedentary and tend to have small home ranges in cities, typically under 1.5 hectares (Havlìćek et al. 2022). With their wide distribution, high reproductive output, and ability to adapt to various urban environments, House Sparrows provide an easy to study and overall excellent model organism to evaluate localized Pb pollution levels and effects on other birds co-inhabiting these areas. Moreover, the localized nature of House Sparrows' habitat makes them promising bioindicators for assessing Pb levels in humans living in Pb-polluted landscapes. In the past decade, there has been a renewed interest in the use of avian bioindicators for assessing Pb risks in humans (Swaileh and Sansur 2006). However, connections between bird and human Pb loads have been sparse beyond that of more-mobile, large home-range species like rock doves (Cai and Calisi 2016). More recent work conducted in an Pb-polluted Australian mining town found that blood Pb levels of House Sparrows correlated with Pb exposures in human children (Gillings-McLennen 2022), creating a more solid link between this sedentary species and human Pb levels. Although further research is needed to determine if the observed adverse physiological or behavioral effects in House Sparrows are also observed in humans living in similar areas, this species clearly has the potential to serve as a reliable indicator of Pb levels and its effects on basic organismal health.

In summary, takeoff flight and movement activity, two behavioral metrics that likely influence fitness outcomes of birds were impaired when captive house sparrows were experimentally exposed to sublethal concentrations of Pb representative of contaminated sites. We have yet to identify the mechanisms by which Pb affects these biomechanical and movement metrics, though it is likely that a suite of physiological and neurological processes are disrupted by the exposure to this heavy metal. The decreases in behavioral performance that we report in this study are likely indicative of what populations of wild birds inhabiting Pb-polluted, anthropogenically-disturbed areas may experience, potentially affecting the evolutionary trajectories of exposed individuals, as well as the surrounding ecological community.

Chapter 2. Investigating Avian Behavioral Adaptations to Pb within a Chronically-Exposed Population of House Sparrows

Introduction

As the world has industrialized over the past two centuries, species of wildlife has encountered chronic anthropogenic stressors that often influence organismal health and fitness. Among these stressors are contamination of environments with lead (Pb); a heavy metal that imposes negative health effects on organisms (Assi *et al.* 2019, Williams *et al.* 2018). Lead is mined for use in paints, electrical components, batteries, plumbing, ammunition, fishing tackle, and until recently, gasoline (Cheng and Hu 2010, Assi *et al.* 2019). Despite stricter regulations and elimination of Pb from some of these products in some parts of the globe (Scheuhammer and Thomas 2014), the emissions and waste associated with manufacture and use of these products has resulted in long-lasting Pb pollution that continues to threaten organisms.

Being an element, Pb does not easily break down into harmless particulates or products, and thus a variety of organisms living in Pb-polluted areas must cope with repeated, long-term exposure to this toxic stressor. For example, evidence has shown that the endangered California condor (*Gymnogyps californianus*) and golden and bald eagles (*Aquila chrysaetos* and *Haliaeetus leucocephalus*) are continually exposed to high concentrations of Pb due their ingestion of carcasses that were shot by hunters using Pb-based ammunition (Finkelstein *et al.* 2012, Pain *et al.* 2019, Slabe *et al.* 2022). In addition to chronic exposure to Pb through ammunition ingestion, Pb pollution from industrial sources also influences wildlife. Small mammals such as white-toothed shrews (*Crocidura russula*) and wood mice (*Apodemus sylvaticus*) that live in areas formerly or actively used in the processing of Pb (i.e. ore smelters) show increased Pb in their tissues and various histological, hematological, and hepatic indicators of reduced health (Sànchez-Chardi *et al.* 2009, Tête *et al.* 2014). In the aftermath of the Flint Water Crisis (2014-2017), American robins (*Turdus migratorius*) that were subjected to chronically heightened Pb concentrations via polluted water runoff exhibited decreased overall body condition after years of chronic Pb-exposure (Zahor *et al.* 2021).

As some wildlife populations are chronically exposed to Pb, it is possible that these populations may adapt mechanisms which reduce the fitness consequences of future Pb-exposure. There is precedence for populations to evolve some degree of tolerance to other environmental toxicants. For example, certain plant (Baker 1987, Pitelka 1988) and invertebrate (Kleks and Levington 1989) species occupying polluted areas have showed some signs of local adaptation and tolerance to local pollutants like sulfates, agricultural-nitrates, and even other heavy metals such as mercury. The Atlantic killifish (*Fundulus heteroclitus*) appears to have evolved resistance to exposure to polychlorinated biphenyls (PCBs) (Di Giulio and Clark 2015). This heritable resistance can be passed down lineages and might render some protection against continued exposure to environmental toxicants (Nacci *et al.* 2002, Meyer *et al.* 2003, Oziolor *et al.* 2019). In these cases, the genes involved appear to influence a signaling pathway linked with preventing physiological damage from a range of toxic compounds (Reid *et al.* 2016). In spite of this evidence, we are not aware of studies that show genetic adaptation to environmental Pb, hence we were interested in exploring this phenomenon at a site that has experienced chronic Pb contamination.

Populations of urban house sparrows living in historically-polluted Australian mining towns have demonstrated some indicators of genetic adaptation to Pb pollution. Sparrows in these mining towns show altered allelic frequency and upregulated expression at loci that are associated with metal ion transmembrane transporters, endoplasmic reticulum function, protein ligase activity, and other phenotypic expression changes expected for organisms under Pb-related physiological stress (Andrew *et al.* 2019). At one of these mining towns, Broken Hill (New South Wales, Australia), the local adaptation of house sparrows to Pb-exposure appears sufficient that various indicators of physiological health (body condition, heterophil/lymphocyte ratio, reticulocyte levels, packed red blood cell volume) do not vary across sites with higher and lower concentrations of Pb pollution (Harris 2022). Such indices are expected to be adversely impacted by exposure to Pb (Cid et al. 2018, Williams et al. 2018, Zahor et al. 2021, Hiraga et al. 2008).

The house sparrows in Broken Hill are good candidates to show local adaptation, as they appear in large numbers, are sedentary with small home ranges, individuals are rarely observed in more than one area of the town, and the populations have resided in the town for at least 100 years and have been exposed to Pb throughout that time (Andrew *et al.* 2017, Harris 2022). However, to further investigate the degree of local adaptation of Pb-tolerance, we are currently missing crucial information about fitness variation in association with Pb-exposure. To get closer to that understanding, the goal of this study is to examine how fitness-related behaviors are related to the spatial distribution of Pb at Broken Hill through sampling birds from sites of higher and lower Pb concentrations.

As we do not know the degree of local adaptation of Pb-tolerance in Broken Hill, we initiated this field study to test two hypotheses representing the extremes of possible explanations for how fitness-related behaviors might relate to Pb-exposure. According to our first hypothesis, the house sparrows have locally adapted to chronic Pb-exposure and, consistent with other studies (Harris 2022, Ali 2023), we would predict no difference in behavioral performance between birds sampled from sites of higher and lower Pb concentrations. Our second hypothesis posits that the sparrows have yet to adapt to local Pb-exposure and we will observe compromised behavioral performance in birds caught from sites of higher Pb concentrations compared to those sampled from sites of lower Pb concentrations. As these hypotheses are at two ends of an adaptive spectrum, it is also possible that we will observe some blend of these predictions, which would be consistent with ongoing but incomplete local adaptation.

Methods

Study site

We conducted this study in the town of Broken Hill, NSW, Australia (31.9596° S, 141.4608° E; Fig. 7). Broken Hill is built over one of the world's largest lead-zinc-silver ore deposits and has been mined for nearly 150 years (Taylor et al., 2014). Due to the mining activities, the soil and surface dust of Broken Hill is contaminated with Pb (mean: 2,450 mg/kg) and zinc (mean: 3,710 mg/kg) (Taylor et al., 2014, Mclennon-Gillings 2022).

Within Broken Hill, there is a clear spatial pattern of Pb in soil and dust (Taylor *et al.* 2014, Mclennon-Gillings 2022), which is generally explained by the direction of prevailing winds in the area—areas downwind of the mining facility are more heavily contaminated than those upwind (Fig. 8). As expected, areas immediately adjacent to the mine in all directions were heavily contaminated regardless of prevailing wind direction (Mclennon-Gillings 2022). This spatial distribution of environmental Pb informed where we sampled house sparrows. House sparrows have resided in Broken Hill for over a century and are abundant across nearly all parts of the town (Andrew *et al.* 2017).

Sample locations

We studied house sparrows that were caught in areas of Broken Hill with known “higher” and “lower” soil Pb concentrations (Fig. 8) (Mclennon-Gillings 2022, Harris 2022, Ali 2023). There is a strong positive association between soil Pb concentrations and concentrations of Pb in the blood of house sparrows in Broken Hill ($r^2 = 0.67$; Mclennon-Gillings 2022). The highly sedentary nature of house sparrows in urban areas, including in Broken Hill, helps to associate birds with specific localities (Swaileh and Sansur 2006). Typically in urban areas like Broken Hill, home and feeding ranges are smaller than an area of $400m^2$ (Havilček *et al.* 2022). Birds caught and sampled in our lower- and higher-Pb sites have been found to have blood-Pb concentration ranges of 3-16 µg/dL in the lower area and 42-74 µg/dL in the higher area (Mclennon-Gillings 2022). The blood-Pb concentrations observed in the higher sites are considered indicators of lethal exposure in other birds and other locations (Franson and Pain 2011, Williams *et al.* 2017) yet these house sparrow populations appear stable (Mclennon-Gillings 2022, Harris 2022).

Sampling and assay protocols

From 23 May - 11 August 2022, we caught house sparrows in mist nets from 7:00 to 15:00 at multiple sites in Broken Hill (Fig. 5b). Immediately after extraction from a net, we performed the in-hand aggression assay (see below).

Following this, we banded all birds with an uniquely-numbered steel leg band (Australian Bird and Bat Banding) and extracted a small blood sample (<50uL) by puncturing of the brachial vein with a 26-gauge needle. This blood sample was collected into a 70µl capacity capillary tube and transferred into a microcentrifuge tube with ethanol for use in other studies unrelated to the work presented in this thesis. Each bird was also processed for morphometric measurements (tarsus length, flattened wing chord, body mass) before entering into the other two behavioral assays.

Assay 1. In-hand aggression

We performed three in-hand aggression assays (struggle assay, breathing assay, agonism assay; n = 538) using the same protocols as described previously in chapter 1. For birds that we re-caught at least 9 days after initial capture, we repeated all three in-hand aggression assays to assess the repeatability of these metrics (*n* = 61).

Assay 2. Escape Takeoff Flight

We performed an escape take-off flight assay for birds that we caught between 28 May 28 – 5 July 5 2022. The takeoff protocols were the same as described in Chapter 1 except that we used a small wooden platform as a takeoff point instead of a perch, and the flights were conducted in an open environment so that birds were free to fly away following their takeoff.

A flight was considered “successful” if the bird jumped from the platform and flew out of the flight studio without stopping. If a bird jumped onto the ground and began hopping around the studio and/or not actively flying, the flight was considered “failed” and was excluded from analysis. Of 256 attempted flights, 204 were classified as successful.

Using established processing protocols and formulas (see Chapter 1) we calculated the total energy expended in the first 15 frames of flight as well as takeoff leap force.

Assay 3. Activity Assay

For birds that we caught between 15 July - 15 August 2022, we conducted activity assays. We quantified activity by placing an individual bird in a novel arena with the same dimensions and characteristics as described in Chapter 1.

To begin an activity trial we placed a focal bird into the acclimation chamber for 5 mins (Fig. 4, McCown *et al.* 2015). All birds entered the naturally-lit activity chamber readily. We video recorded all movements of the focal bird for 8 mins on a GoPro Hero7 camera (60 frames per second, 1040p resolution). From these videos, we quantified the frequency of 5 behaviors associated with activity, as described in Chapter 1.

At the end of each activity trial, the roof of the activity chamber was raised and the bird flew back into the environment. At the end of each day of approximately 7-8 trials, we cleaned the activity chamber to minimize cues left from repeatedly using the apparatus.

Statistical Methods

To assess normality in the error distribution of our data, we performed suitable Shapiro-Wilks tests. Based on whether the assumption of normality was maintained, we would run either a parametric Welch's t-test or a non-parametric Mann-Whitney U-test to compare differences between the means of the higher and lower Pb groups.

To compare the in-hand aggression metrics of struggles, average breathing, and agonism between higher and lower Pb birds we similarly conducted either a Welch's t-test or a Mann-Whitney U test. Specifically, we used a Welch's t-test for the normally-distributed average breathing rate and Mann-Whitney U-tests for the struggle and demeanor metrics.

To assess the effect of environmental Pb concentration on escape takeoff flight performance, we compared the average energy expended during takeoff and takeoff leap force between birds caught in the lower Pb area to those in the higher Pb area. Based on normality, we used a Welch's t-test to compare average energy expended during flight and then a Mann-Whitney U test takeoff leap force.

In order to examine movement activity levels within our birds, we reduced the dimensionality of the five behavioral metrics of the activity assay using principal components analysis (PCA). The PCA rendered two interpretable principle components (see Results for descriptions) to be used in comparisons. We compared between the PC1 and PC2 scores between higher and lower Pb birds by using Mann-Whitney U-tests.

All analyses were performed in R version 4.2.1 (R Development Core Team, 2021) interpreting two-tailed tests of probability.

Results

In-hand Aggression assays

In birds that we captured and assayed on two separate occasions, we found evidence for within-bird repeatability of one of the metrics, breathing rate (r^2 = 0.754). Neither the number of struggles ($r^2 < 0.1$) nor agonism score ($r^2 < 0.1$) appeared repeatable across these duplicate measurements.

Higher ambient Pb concentrations were associated with variation in one of these three in-hand aggression metrics (Fig. 9). Birds caught in the higher Pb area exhibited a lower average breathing rate than those caught in the lower Pb area (Welch's, $t = -5.04$, $n = 538$, $p < 0.001$). However, neither the number of struggles (Mann-Whitney, $w = 36936$, $n = 538$, $p = 0.553$) nor agonism score (Mann-Whitney, $w = 35186$, $n = 538$, $p = 0.658$) varied consistently between birds caught in the higher or lower Pb sites.

Escape takeoff flight assay

Heightened exposure to Pb was associated with decreased escape takeoff performance (Fig. 10). In particular, house sparrows caught in the higher Pb sites expended less energy during takeoff than those caught in the lower Pb site (Mann-Whitney, $u = 4081$, $n = 204$, $p = 0.008$). Similarly, Pb influenced takeoff leap force (Welch's, $t = -3.008$, $n = 204$, $p = 0.003$) such that sparrows caught in the higher Pb sites exerted less force in their takeoff leap than those caught in the lower Pb area.

Activity assay

The PCA of the five activity metrics returned two PCs, which collectively explained 76% of the original variation (Fig. 11a, Table 3). PC1, which we interpreted as total “movement activity”, explained 56% of the variation and was positively loaded with all 5 behaviors. PC2, which we interpreted as “sedentary self-maintenance”, explained an additional 20% of the variation. Sedentary self-maintenance was negatively loaded with “self-maintenance” and positively loaded with “areas visited”. There were no consistent differences in either PC1 scores (Fig. 11b; Mann-Whitney, w = 2973, n = 153, p = 0.924), Fig. 8b) or PC2 scores (Fig. 11c; Mann-Whitney, w = 2737, n =153, p = 0.347, Fig. 8c) in birds caught from higher Pb sites compared to those caught from lower Pb sites.

Discussion

Highlighting the complex state of the proposed Pb adaptation on the behavior of Broken Hill's House sparrow population, we found evidence in support of both of our hypotheses. The decreases in escape takeoff performance and reduced breathing rate indicates that birds exposed to more Pb still suffer from a potential fitness deficit. However, there was no association between Pb-exposure and our assays of activity, or the struggling or agonism assays, suggesting a degree of local adaptation to the Pb in specific areas of Broken Hill.

We found that birds captured in the higher Pb sites showed a 6.5% decrease in the average energy expended over their escape takeoff flight, and a 12% decrease in the takeoff leap force. As the energy gain metric we used is a summation of kinetic and potential energy (Williams and Swaddle 2003, Swaddle *et al.* 1999), birds expending less total energy during their takeoff flight moved more slowly and gained less vertical height during takeoff. Similarly, the reductions in takeoff leap force, an negative effect size of 12.8%, implies those birds generated less force from their leg muscles as they jumped off the starting platform (Provini *et al.* 2012, Bonser and Rayner 1996). Taken together, the reductions that we observed here are indicative of an overall slower escape flight performance that gained less height from the ground.

It is likely that an escape takeoff that is slower, less forceful, and results in the bird gaining less height from the ground would make that individual more susceptible to depredation (Metcalfe and Ure 1995, Witter *et al.* 1994, Swaddle *et al.* 1999). Rapid, unimpaired takeoff from the ground is often crucial for small birds in evading both ground-based and aerial predators (Veasey *et al.* 2001, Jones *et al.* 2009, Witter *et al.* 1994). In addition to anti-predator evasion, decreased flight performance may have detrimental consequences for other avian activities such as seasonal migration (Seewagen 2020, Perez *et al.* 2007), food provisioning (Crino *et al.* 2017), reproductive output (Byers *et al.* 2010), and resource competition (Altshuler 2006, Ecke *et al.* 2017). Consequently, the decreases in takeoff flight performance that we see in birds from the higher Pb sites are likely linked with lower avian fitness.

We also found that the birds in the higher Pb sites demonstrated a significant 6.8% (on average) slower breathing rate when being handled than birds caught in the lower Pb sites. Given that all birds would be stressed by capture and handling, we interpret this slower breathing rate to be a manifestation of a Pb-induced reduction in muscular and physiological performance—which is consistent with the reduced takeoff flight performance. In addition, breathing rate has been associated with metabolic respiration, due to the influx of oxygen on each breath (Markley and Carrier 2010). As breathing rate and metabolic respiration can be positively correlated (Markley and Carrier 2010, Millidine *et al.* 2008, Gallivan and Best 1980), the slower breathing rate might be indicative of a slower metabolic rate in birds exposed to high Pb levels. Accordingly, depressed metabolic rates have been proposed to decrease individual fitness and survival for birds living in polluted areas (Di Giulio and Scanlon 1985, Koivula *et al.* 2011, Seewagen *et al.* 2022). It is also possible that the reduced breathing rate of birds from the high Pb sites could indicate a compromised or a less perceptive stress response to capture and handling. It is very possible that Pb may interacts with various systems involved in regulating and activating the HPA axis of birds, influencing their ability to mount an adaptive stress response (Baos *et al.* 2006, Eeva *et al.* 2005, Fritsch *et al.* 2019, White *et al.* 2022). Similarly, Pb's known interferences on the avian neurological system may impair the higher Pb's birds' ability to perceive danger and react in the same manner as an unexposed birds (Nam *et al.* 2012, De la Casa-Resino *et al.* 2015). Under any of these interpretations, the birds that are exposed to more Pb are likely experiencing a deficit that would influence their fitness.

In addition to observing these Pb-related potential fitness deficits, we also report contrasting evidence that the birds from the higher Pb sites had equal performance to birds from the lower Pb sites in terms of activity, struggling, and the in-hand agonism assessment. We were particularly surprised to see a lack of difference in the activity assay between birds caught from the higher and lower Pb sites. The observed differences in takeoff and breathing rate suggest an overall suppression of muscular activities and/or performance due to Pb exposure, but that was not manifested in the birds' overall movements in the activity arena. Our own studies (see Chapter 1) and those of others (Grunst *et al.* 2018) indicate that exposure to Pb often leads to reductions of activity metrics such as the number of flights, hops, and other behaviors associated with movement. It could be that our activity arena was too novel and thus the metric we quantified was more related to exploration or perception of a novel environment rather than activity (Sánchez-Tojar *et al.* 2017, Poblete *et al.* 2021, McCown *et al.* 2015). Also, the escape takeoff flight and breathing rate assays may be more reflective of maximal performance under stress, whereas movements in the activity arena may not reflect performance at the same sort of limit. It is also possible that the degree of local adaptation needed for optimal performance in the takeoff flight and breathing assays is incomplete and, hence, not manifested in general movement/activity metrics.

Consistent with incomplete local adaptation, we also saw no difference between birds caught in the higher and lower Pb sites in terms of the in-hand struggle and agonism assays. We expected that both of these assays would give us insight into the relative aggression of individual birds (Brommer and Kluen 2012, Class and Brommer 2020) and predicted that increased exposure to Pb would alter aggressive responses. We did not see that. However, these interpretations must be couched within the observation that our repeated measurements of these assays were not correlated with each other. In other words, there was a lot of variation within individuals' repeated responses for these two metrics. This makes it harder to detect and interpret between group (i.e. higher vs lower Pb sites) differences in either assay. Given that many previous studies have reported Pb-related shifts in avian behavior (McClelland *et al.* 2019, Janssens *et al.* 2003, Sato *et al.* 2022), it seems quite likely that we did not have the degree of precision to detect a small effect size.

Considering our results holistically, we have evidence that the house sparrow population in Broken Hill is on its way to local adaptation but have not reached a point where the birds in the sites of higher Pb-contamination have equal behavioral performance, and inferred fitness, to those in the less contaminated areas of the town. That interpretation is potentially important to consider in other cases of environmental pollution. The house sparrows at Broken Hill are a great candidate for rapid local adaptation, as they are abundant, site-faithful with small home ranges, have existed there for over a century, appear to breed readily in all areas of the town, and are exposed to a spatially well-defined gradient of Pb pollution (Andrew *et al.* 2017, Harris 2022). If birds in this situation have yet to fully adapt to cope with their Pb-exposure, then we would predict that other situations where bird populations might not be so spatially constrained and/or not exposed to the pollutant consistently for such a long period of time, we would likely fail to see strong local adaptation to toxicants. Birds that undergo migratory journeys and are exposed to contaminants only briefly during the year would be less likely to develop such strong adaptations to more-localized pollutants. Other traits more impactful for their life history including breeding ground quality (Norris and Marra 2007), migratory connectivity (Webster *et al.* 2002), population dynamics (Rushing *et al.* 2016) and global climate change (Webster *et al.* 2002) may receive stronger selection. A similar idea could be applied to birds with larger home ranges that are more reliant on continued resource availability (Monserrat *et al.* 2013) or consistent habitat quality (Marzluff *et al.* 1997) instead of needing to cope with specific local stressors. Conversely, urban birds like the Broken Hill house sparrows that are unable to avoid human-imposed selective pressures may be more likely to adapt to them (Chace and Walsh 2006). In essence, the ecology of the bird species may be a more determining factor in the scale and scope of pollutant-based adaptations.

To assess the overall degree of adaptation to Pb-exposure it would be relevant to compare the performance of birds from Broken Hill to those caught in a town where there is minimal or no Pb-contamination. Our current comparison is limited in that all birds have exposure to Pb in their environment. As such, the birds in the lower-contamination sites are likely adapting to the challenges imposed by exposure to Pb, making it impossible for our study to assess whether adaptation is happening per se and restricts our questions to the degree of local adaptation across a Pb gradient where all sites are Pb-contaminated. Our conclusion that there is partial local adaptation along that gradient does not preclude that there is significant adaptation overall.

Going forward, an important direction would be to generate experimental evidence that further assesses possible adaptation to Pb-exposure at Broken Hill (and other similar sites). It would be highly relevant to design a exposure study (similar to Chapter 1) where study subjects were drawn from the population of adapted house sparrows at from Broken Hill and from towns where there is no (or minimal) Pb contamination. In this design, we could generate experimental evidence to show that the Broken Hill sparrows with their discrete genetic signatures towards Pb resistance (Andrew *et al.* 2019) indeed perform differently than unadapted sparrows that lack this resistive adaptation. By utilizing a captive exposure method, this design would also allow for control of the amount of Pb that the birds would be exposed to, allowing us to be more certain of Pb's role in driving the local adaptation seen in Broken Hill. Importantly, our results could help inform this next step. Based on the observational results of this study, we can be more confident that the local Pb adaptation in Broken Hill's sparrow population is incomplete and that all traits negatively affected by Pb like takeoff flight and breathing rate may not show resistance to Pb exposure. As such, this study may point to assessing behavioral traits that may be more subtly impacted by Pb such as movement activity. Should the experimental exposure study find evidence of decreased behavioral performance in unadapted birds exposed to Pb than that of the Broken Hill birds also exposed to Pb, then we can be more certain that the genetic adaptations seen in this population are indeed a result of Pb-induced selection.

In summary, the Broken Hill sparrows show partial behavioral adaptation to local Pb pollution. Birds in the high Pb area demonstrated expected decreases takeoff flight performance and average breathing rate, two behavioral metrics that likely influence fitness outcomes of birds. Meanwhile, there was no associations between Pb-exposure and our assays of activity, or the struggling or agonism in-hand assay across the Broken Hill Pb gradient. This lack of significance may point to local adaptation having acted upon some of the mechanisms used in birds’ behavioral responses during these assays. While our results are limited, the assessment that local adaptation in Broken Hill has partially occurred after just over a century of heightened Pb exposure may suggest that the unique ecology of the House Sparrow has made them more likely to undergo selection to this stressor. As such, other birds with larger home ranges or migratory life histories may not be able to adapt to this anthropogenically-exacerbated challenge. Future work should consider utilizing a captive exposure study between the birds of Broken Hill and unadapted birds to generate experimental evidence that further assesses possible adaptations to this ecological stressor.

CONCLUSION

In conclusion, this set of studies was able to shed further light on the ways that Pb influences the behavior of a globally-distributed, commensal bird species, while also providing more directions for the focus of future work. Taken together, this work has generated a unique set of findings that adds to the literature on behavioral ecotoxicology.

As per our results of our captive work, we were able to generate experimental evidence showing Pb-incurred deficiencies in flight performance and movement activity; behaviors vital for avian survival. We importantly find this using the House Sparrow, a species heavily linked with the same anthropogenically-disturbed environments in which Pb pollution is most commonly associated. To the best of our knowledge, this is the first study that has experimentally shown that this human-commensal species may have key behavioral responses altered by Pb pollution. Importantly, our exposure methods have provided an apt framework and for allowing us to study the costs and benefits of adaptation in sparrow populations potentially adapting to the fitness challenge imposed by Pb; such as those in Broken Hill.

Our findings in Broken Hill are subject to multiple lines of interpretation based on the incomplete nature of the proposed adaptation to Pb. Given that this is a wild population and we could not quantify the different selective pressures these birds are facing, it is tough to fully contextualize the role Pb plays in driving adaptation to the behaviors we examined. The selective forces dictating which behaviors are most important for survival within the local population of sparrows at Broken Hill may have spurred the development of resistance at an inconsistent rate generating the mixed effects on behavior we observed. While unlikely due to the presence of ground-based predators across the town, if there are less non-aerial threats, there may be less of a need to adapt resistance as to impact takeoff flight parameters, or, quantifiable differences in breathing rate within the context of capture stress. Or perhaps the mechanisms involved in takeoff flight performance and/or breathing rates are more deeply impacted by the presence of high Pb making developing such resistance much more complex within the relatively short timeframe these birds have been exposed to high Pb levels. Furthermore, perhaps we are witnessing a tradeoff spurred by an inequal rate of adaptation; as the ability to avoid predation is more complexly impacted by Pb, these birds may have developed increased perceptive abilities or other traits to better compensate. Based on the results of our captive, it is clear that takeoff flight and movement activity are subject to Pb's negative effects in naïve birds so further study is needed to understand the selective forces at play that allow us to observe the non-analogous decreases in behavioral trends we report.

While we saw decreases in takeoff flight performance in both the captive and field studies, the effect sizes were not equal, giving further credence towards the Broken Hill birds' adaptation. If high Pb levels had been influencing both of these populations in the same way, we would expect similar effect sizes across treatment groups; something this work does not support. Instead, the decrease in escape flight energy across the Pb areas in Broken Hill was just 6.5% and this is much lower than the 21.9% treatment-average decrease for the naïve, captive birds. Similarly, the reduction takeoff leap force of 12.8% exhibited in the field study was notably smaller than the 50.2% treatment-average decrease seen in the captive study. Lastly, it is important to note that the Pb exposure levels, as well as bird blood Pb levels, are much higher in Broken Hill than the levels we simulated in our captive study. This makes the lack of an analogous effect size based on individual Pb levels yet more evidence to point towards some kind of resistance within this population. As such, it is important to note that while our work finds decreases in takeoff flight performance within the Broken Hill sparrows, Pb's effect does not seem to be a driving trends nearly as strong in this population as our captive study; also supporting the assessment of incomplete adaptation within the Broken Hill sparrows.

The results of the proposed captive study involving the Broken Hill birds, as well as future behavioral work within the Broken Hill sparrow system should give us further insights into the costs and benefits of adapting to Pb pollution. We hope that this collective work provides more evidence into how organisms may be affected by, or cope with, the human-imposed challenges that have become hallmarks of the Anthropocene.

APPENDIX

Aggression Assay Type	Control Mean (+/-SE)	Lower Mean (+/-SE)	Higher Mean (+/-SE)	Shapiro Wilks' p-value	n	Test Statistic	Test Result
Number of Struggles	3.529 +/-0.591	2.833 +/-0.575	2.647 +/-0.512	$p < 0.05$	51	$x^2=0.808$	p=0.668 (KW)
Average Breathing	68.735 +/-1.451	66.250 +/-1.995	71.176 +/-2.230	$p > 0.05$	51	$F_2=0.564$	p=0.573 (ANOVA)
Agonism Score	2.2941 +/-0.155	2.278 +/-0.178	2.118 +/-0.171	$p < 0.05$	51	$x^2=0.417$	p=0.812 (KW)

Table 1. In-hand aggression results from captive study. Values are summarized and means are divided by treatment (Control/lower/higher). A shapiro test was run on the resulting residuals of an ANOVA to determine normality; if a $p > 0.05$ was attained and thus the sample was relatively normal, the ANOVA was maintained. Otherwise, a Kruskal-Wallis (KW) rank-sum test was run as a nonparametric assessment. All three variables did not vary significantly between treatments and thus post-hoc tests were not conducted.

Behavior/ Variable	PC1 (69%)	PC2 (18%)
Total Flights	0.899	-0.267
Total Hops	0.910	0.333
Perches Visited	0.864	-0.120
Areas Visited	0.763	0.604
Total Self-maintenance	0.697	-0.603

Table 2. First and second principal component from the captive study’s activity assay. The percentage of variation explained is listed next to each. PC1 is associated with positive directionality of all five metrics. PC2 is most positively correlated with areas visited and most negatively related to total self-maintenance.

Behavior/ Variable	PC1 (69%)	PC2 (18%)
Total Flights	0.0.836	-0.029
Total Hops	0.853	-0.016
Perches Visited	0.839	-0.008
Areas Visited	0.826	0.115
Total Self-maintenance	0.853	-0.016

Table 3. First and second principal components from the Broken Hill study's activity assay. The percentage of variation explained is listed next to each. PC1 is associated with positive directionality of all five metrics. PC2 is most positively correlated with areas visited and most negatively related to total self-maintenance.

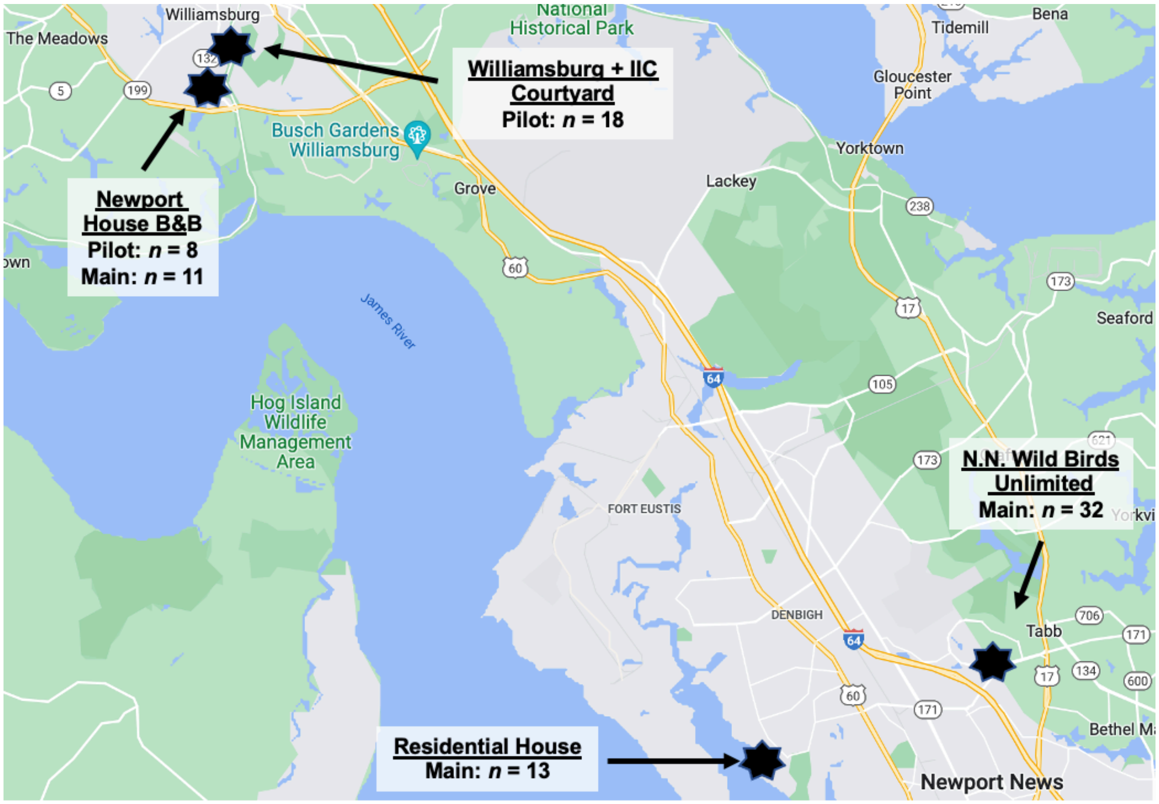

Figure 1. Map of House Sparrow Catching Sites within Hampton Roads region of Virginia, USA. Stars indicate approximate locations of where mistnetting for House Sparrows occurred during February-March, and September-November 2022. Text boxes give a brief descriptor of location, and indicate how many individual sparrows were caught at sites for either the “pilot” studies (Feb-Mar), or the main captive study (Sept-Nov).

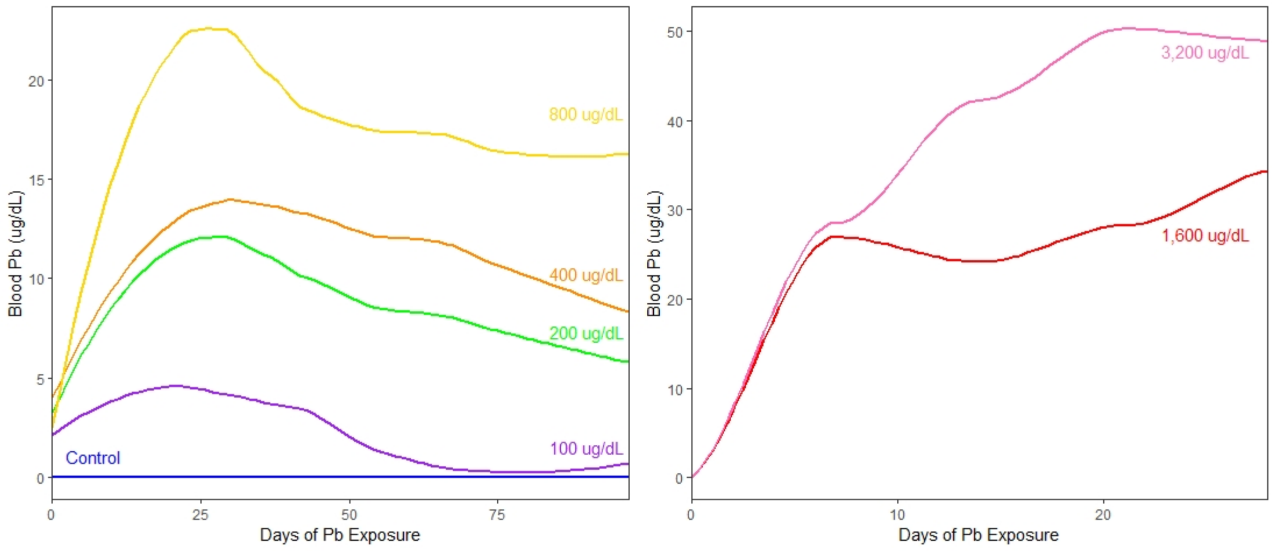

Figure 2. Pilot 1 and 2 House Sparrow blood Pb levels. Figure 2a represents the average blood Pb level of house sparrows exposed to Pb at the four pilot 1 concentrations while figure 2b does the same but for the two concentrations in pilot 2. Lines symbolize the moving averages in these levels over time. For all pilots, sampling occurred on a weekly basis. Exact Pb/DI water concentrations are indicated along the corresponding line of change.

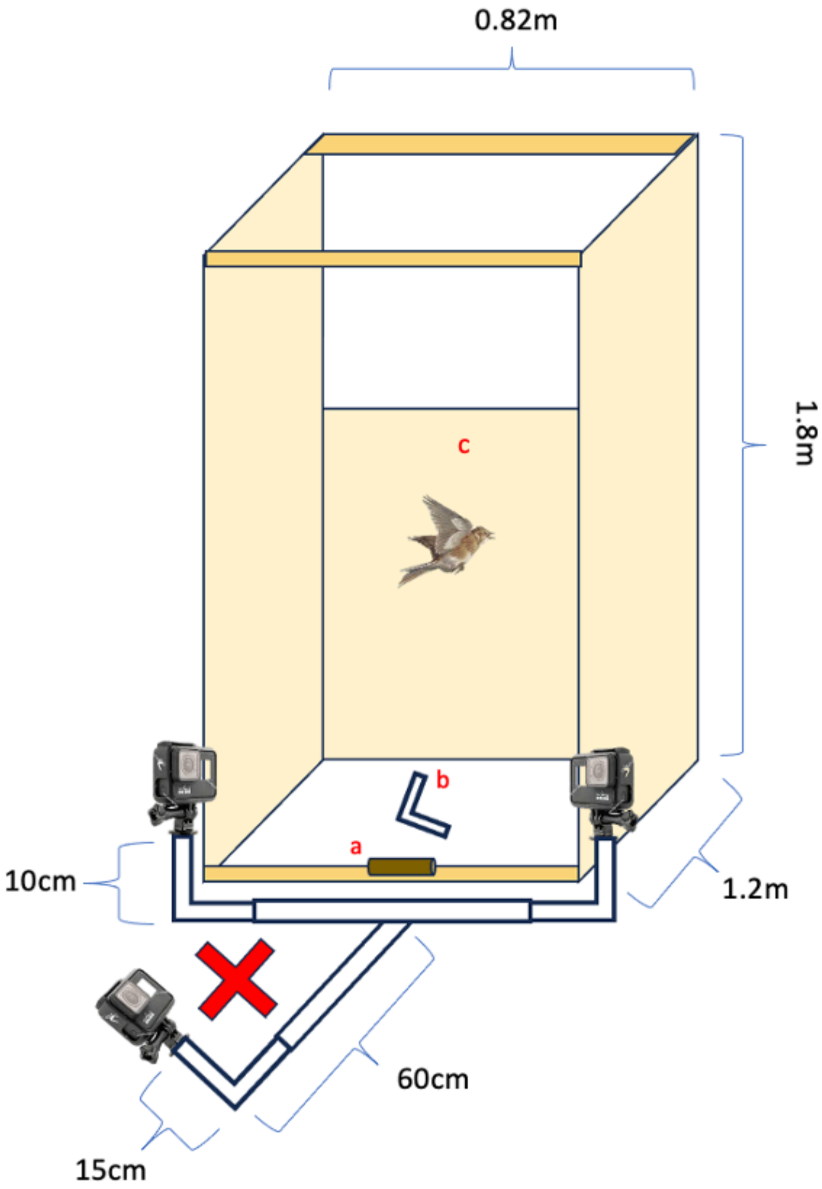

Figure 3. Diagram of “flight studio”. A schematic of the apparatus used in the takeoff flight assay. A bird would be first placed on wooden perch (a) in a secured hold by an experimenter who would be positioned in the place of the red “X”. Upon release, the bird would fly through the studio and over the barrier board (c). There was nothing in the studio besides a small, “L”-shaped PVC structure (b) that aided in processing. Flights were recorded on three GoPro cameras-all in different positions-held on a fixed stand.

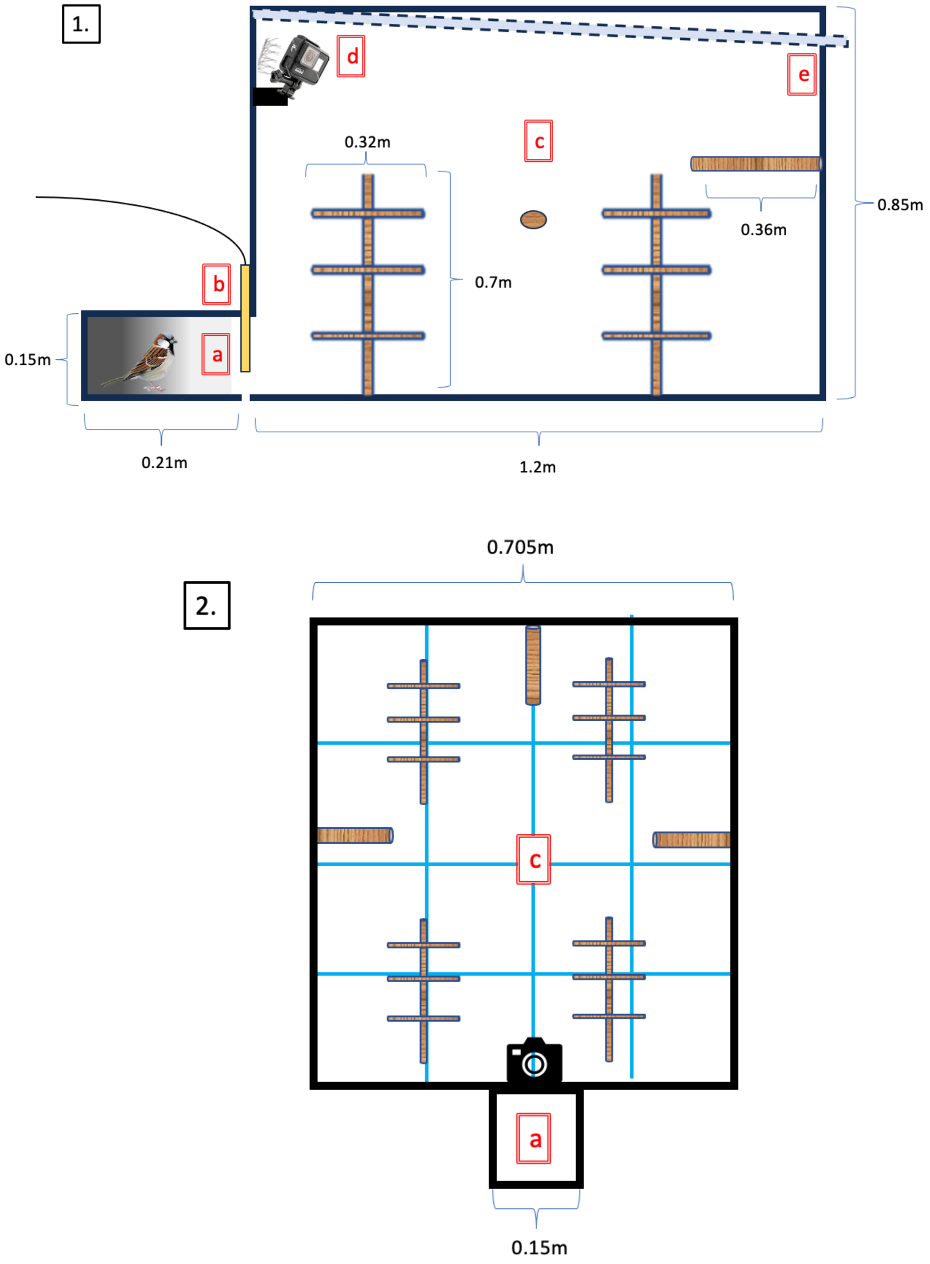

Figure 4. Diagrams of “novel environment” used in activity assay. A schematic of the apparatus setup from a side view (1) and a top-down view (2). The bird was first placed in a darkened acclimation chamber (a) for 5min. After this, the experimenter remotely removed the acclimation chamber door (b) and the bird entered a well-lit novel environment (c). The door immediately was dropped back into place so as the bird could not re-enter the acclimation chamber. Birds had 8 minutes to move around the novel environment and this was captured on a raised GoPro Hero7 (d) that could visualize the entire environment. The ability to see the entire environment was aided by a down-slanted roof made of 1 1.5m long panel of translucent corflute.

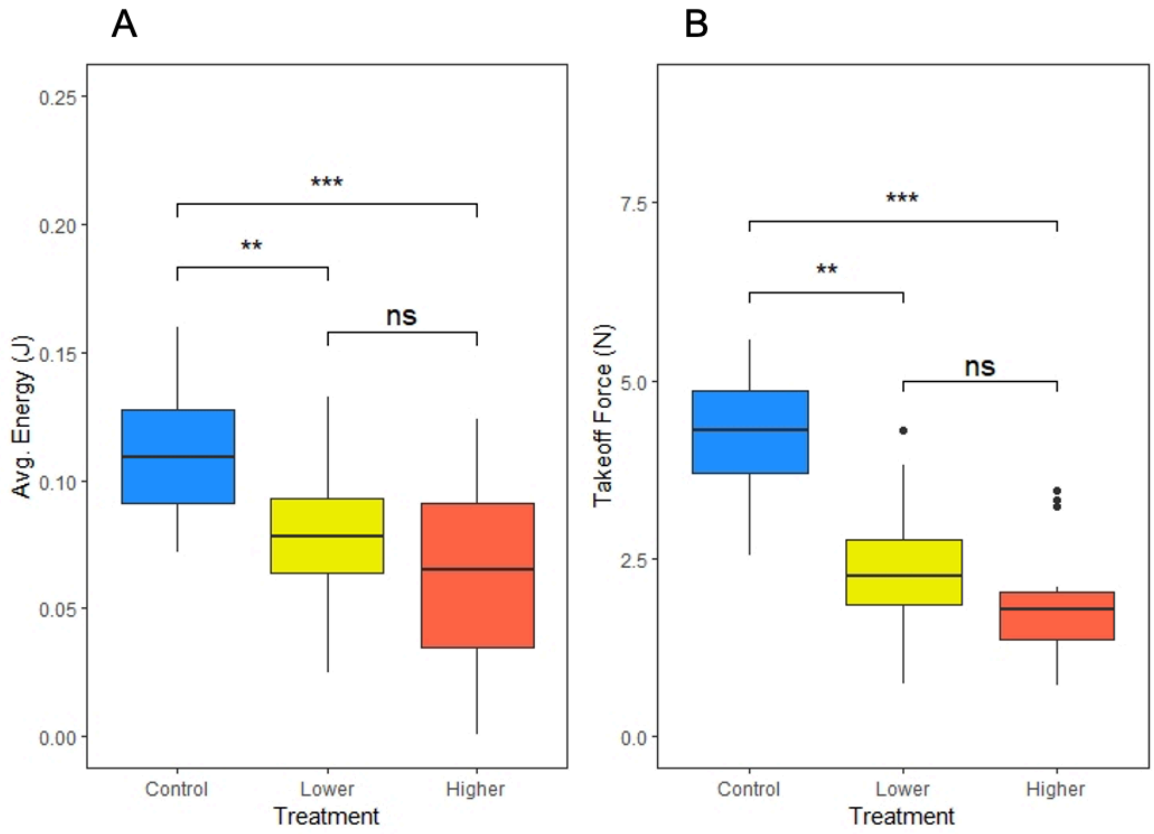

Figure 5. Comparison of takeoff flight performance metrics based on sublethal Pb exposure. Data was analyzed with an ANOVA and Tukey HSD after meeting assumptions (n = 51). Boxplots represent the distribution of data of average energy expended (5a) or takeoff leap force (5b) across the 3 treatments. The black bar inside the boxplot represents the median of data, the limits of the boxes represent the IQR, and the lines extending from the boxes represent the extremities of the data. Black dots are outliers of the data. “ ** ” shows a comparison that is significant to the $p < .001$ level and “ *** ” shows a comparison that is significant to the $p < .0001$ level. “ns” represents a comparison in which $p > 0.05$.

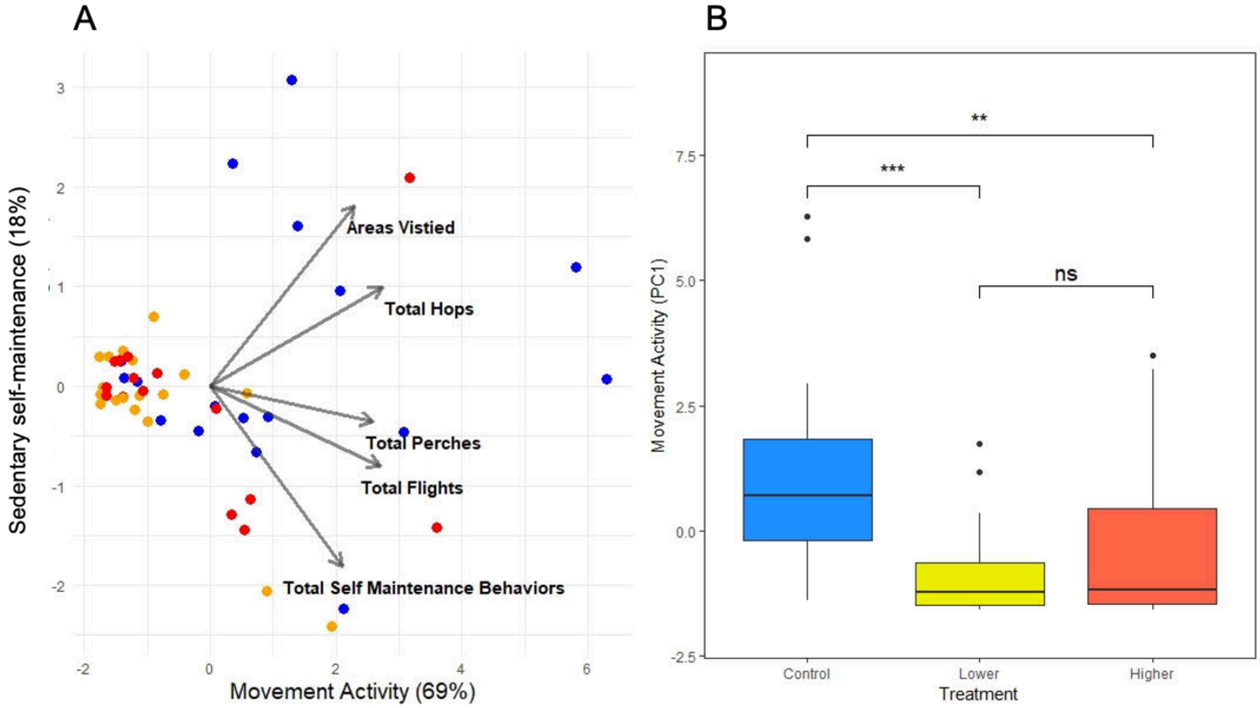

Figure 6. Principal component analysis and comparison of PC1/“movement activity” scores. Figure 6a represents the comparison of PC1 scores (Movement activity) by PC2 scores (Sedentary self-maintenance) from a principal component analysis comprised from 5 behaviors: total flights, total hops, total perches, total areas, total self-maintenance behaviors (n = 51). PC1 comprises 69% of the data while PC2 comprises an addition 18% so that these two variables together encapsulate 89% of the data. As the 5 loading factors, visually represented by the arrows, are all positively correlated with PC1, a higher PC1 score indicates a bird that is more readily moving around and visiting more disparate locations during it’s trial. Colors represent the treatment of the bird (Blue=control, yellow = lower, red = higher).

Accordingly, figure 6b is a boxplot displaying the data from PC1/movement activity by treatment. Boxes represent the distribution of movement activity across the 3 treatments, the black bar inside the boxplots represents the median of data, the limits of the boxes represent the IQR, and the lines extending from the boxes represent the extremities of the data. Black dots are outliers of the data. Data were analyzed with a Kruskal-Wallis test and Dunn post-hoc assessment. “ ** ” shows a comparison that is significant to the $p > .001$ level and “ *** ” shows a comparison that is significant to the $p > .0001$ level. “ns” represents a comparison in which $p > 0.05$

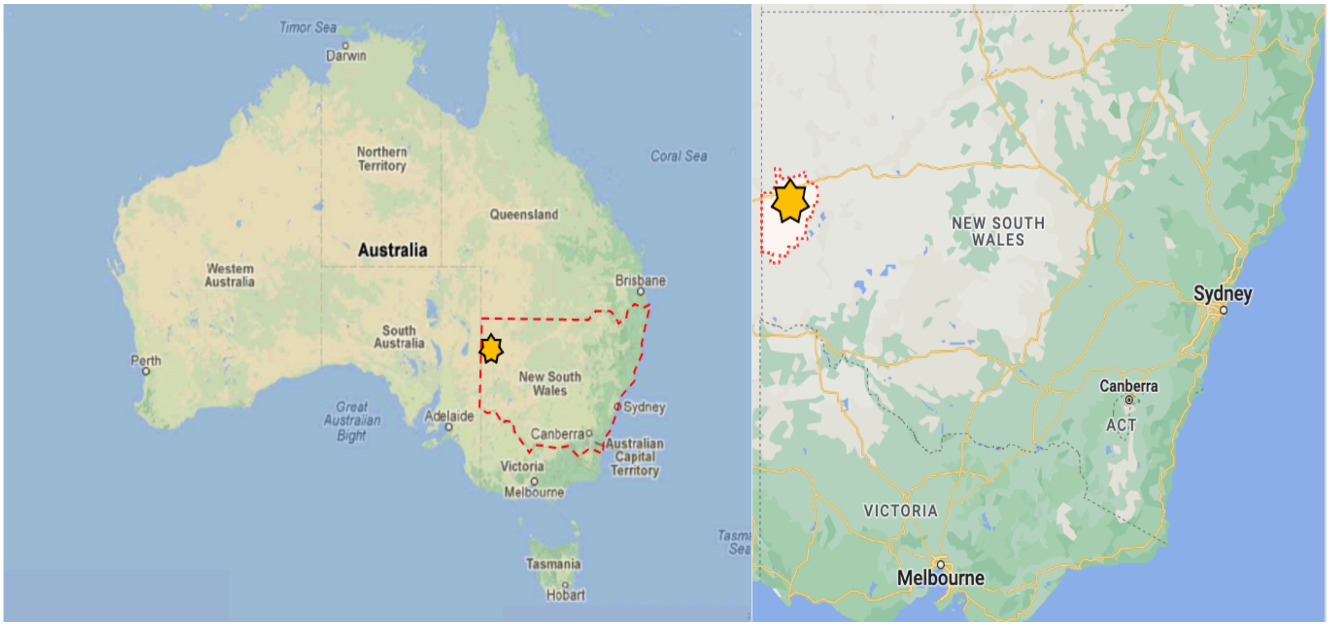

Figure 7. Map of Broken Hill's location within New South Wales and Australia. The map on the left highlights the state boundaries of New South Wales (Red dashed line) and the city of Broken Hill (Orange star) location within Australia. The map on the right shows the city limits of Broken Hill (Red-dashed line) and the main part of the city itself (Orange star) within the state of New South Wales.

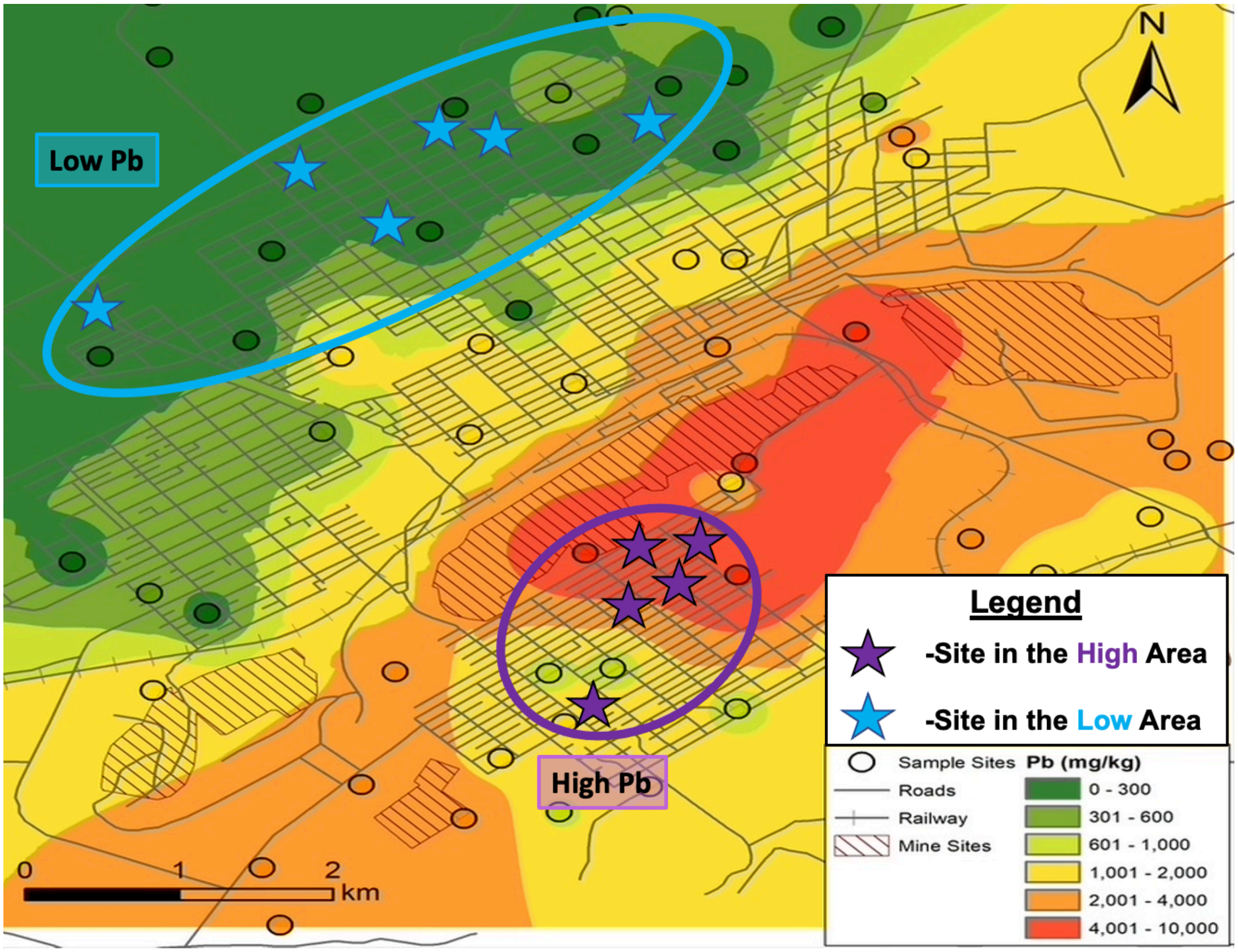

Figure 8. Map of Broken Hill Pb levels and catching sites. A heatmap of soil Pb levels taken from Broken Hill, NSW 2020-2021 (Mclennon-Gillings 2022) overlayed with our study areas and catching sites. Warmer colors represent higher levels of ambient Pb pollution while deeper green colors represent a lack of Pb presence, or lower levels in general. The blue icons represent what we considered the “low” Pb area (circle) and the specific catching sites where we caught at least 1 individual bird (stars). The deep purple icons represents the same concepts except for the “high” Pb area.

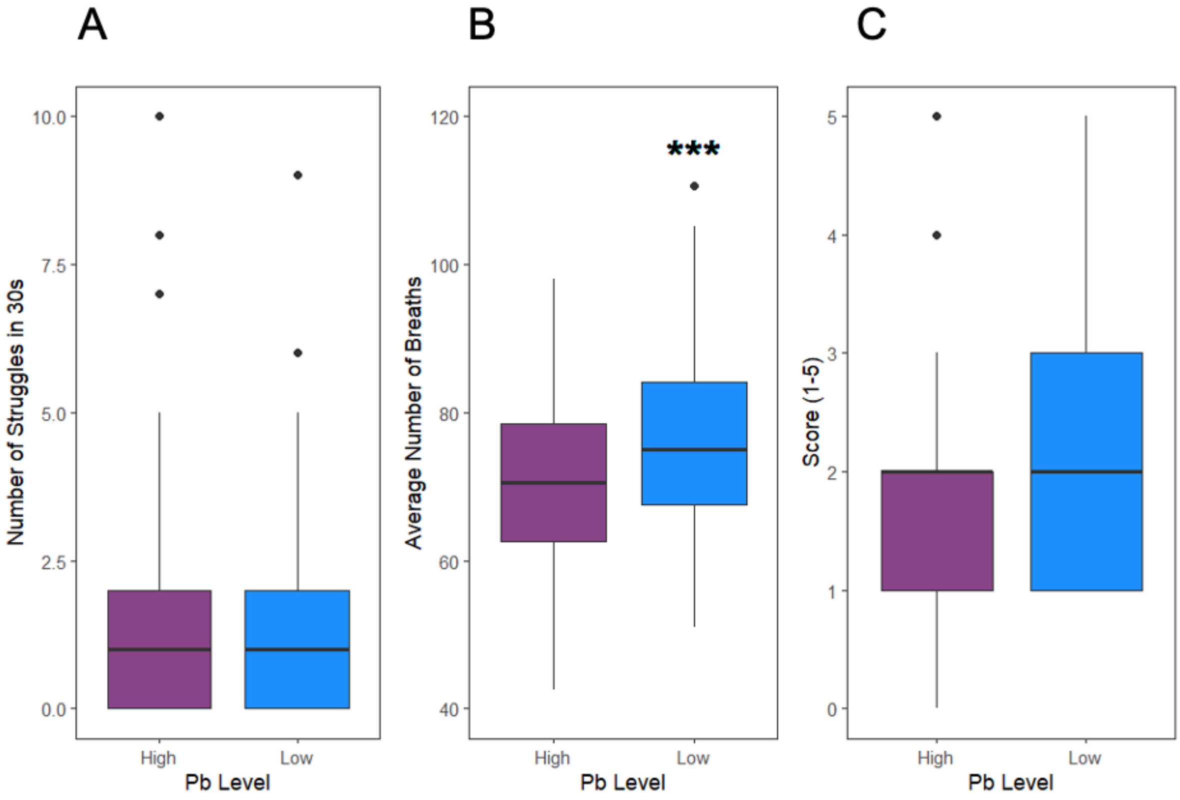

Figure 9. Comparisons of in-hand aggression metrics among high/low Pb birds. All three boxplots showcase separate in-hand aggression metrics among high and low Pb level birds (n = 538). Figure 9a compares the number of struggles, figure 9b compares the average number of breaths/breathing rate, and figure 5c compares the agonism score. Boxes represent the distribution of movement activity across the Pb levels, the black bar inside the boxplots represents the median of data, the limits of the boxes represent the IQR, and the lines extending from the boxes represent the extremities of the data. Black dots are outliers of the data. Data were analyzed with a Welch's t-test (Figure 9b) or a Mann-Whitney U-test (Figures 9a and 9c). " *** " shows a comparison that is significant to the $p > .0001$ level.

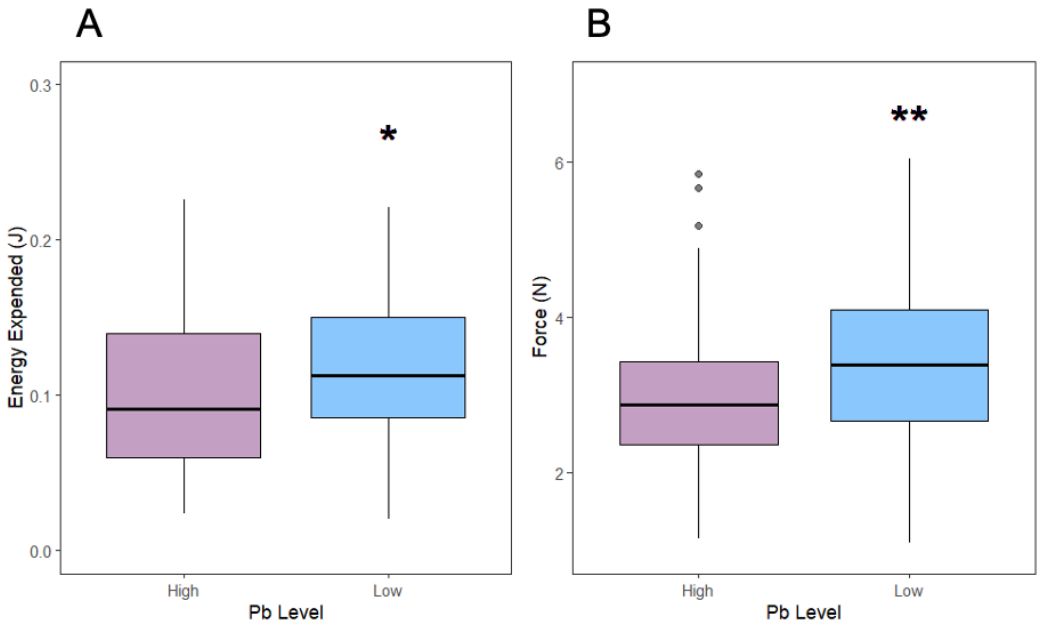

Figure 10. Comparisons takeoff flight performance metrics among high/low Pb birds. Both boxplots showcase takeoff flight performance metrics among high and low Pb level birds (n = 204). Specifically, figure 10a compares the energy expended over the flight while figure 10b compares the leap force exerted. Boxes represent the distribution of movement activity across the Pb levels, the black bar inside the boxplots represents the median of data, the limits of the boxes represent the IQR, and the lines extending from the boxes represent the extremities of the data. Black dots are outliers of the data. Data were analyzed with a Welch's t-test (Figure 10b) or a Mann-Whitney U-test (Figures 10a). " * " shows a comparison that is significant to the $p < .05$ level and " ** " shows a comparison that is significant to the $p < .001$ level.

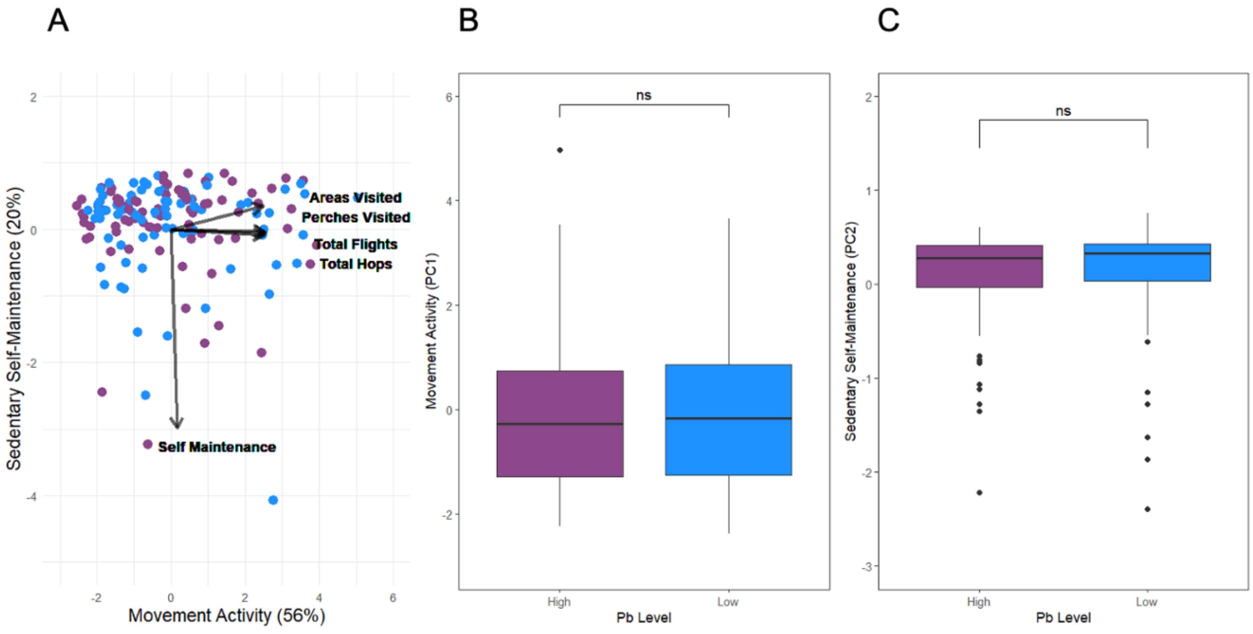

Figure 11. Principal component analysis and comparison of PC1+PC2 scores. Figure 6a represents the comparison of PC1 scores (Movement activity) by PC2 scores (Sedentary self-maintenance) from a principal component analysis comprised from 5 behaviors: total flights, total hops, total perches, total areas, total self-maintenance behaviors (n = 155). PC1 comprises 56% of the data while PC2 comprises an addition 20% so that these two variables together encapsulate 76% of the data. As the 5 loading factors, visually represented by the arrows, are all positively correlated with PC1, a higher PC1 score indicates a bird that is more readily moving around and visiting more disparate locations during its trial. PC2 is negatively correlated self-maintenance and positively correlated with areas visited and perches visited. Colors represent the Pb levels where the bird was caught and assayed (Blue = lower Pb, purple = higher Pb).

Figure 6b and 6c are boxplots displaying the PC scores from movement activity and sedentary self-movement across Pb areas, respectively. Boxes represent the distribution of each PC score, the black bar inside the boxplot represents the median of data, the limits of the boxes represent the IQR, and the lines extending from the boxes represent the extremities of the data. Black dots are outliers of the data. Data were then analyzed with an Kruskal-Wallis test and Dunn post-hoc assessment. “ns” represents a comparison in which $p > 0.05$.

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