The physiological effects of high blood lead levels in the House sparrow

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Declaration

I declare that this thesis, as a whole or in parts, has not been submitted for a higher degree to any other university or institution. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

I wish to acknowledge the following assistance with the research detailed in this thesis:

This project forms a part of an ongoing project on a House sparrow population in Broken Hill under researchers at Macquarie University. I collected haematological samples in 2020 and 2021 that formed the basis of this project. I collected samples in 2020 with field assistance from Dr Riccardo Ton, and 2021 with field assistance from Dr Laura Hurley. I performed all sample processing with assistance and guidance from the microbiology lab staff at Macquarie University, and under the supervision of Professor Simon Griffith. I performed the data analysis with guidance from Callum McDiarmid. I created the figures (with the exception of 2 site maps provided by Max McLennan-Gillings), and prepared the manuscript with guidance from Professor Simon Griffith.

All research carried out for this thesis was approved by Macquarie University Animal Ethics Committee, Animal Research Authority (AEC reference Number: 2020/011-4).

Tiarne Harris Date: 18/02/2022

This thesis is formatted as a manuscript for submission to *Science of the Total Environment* with some exceptions to meet the requirements of Macquarie University. This includes the requirement for an abstract of 200 words, 2cm margins, 1.5x line spacing, and figures and tables embedded within the text.

General acknowledgements

Firstly, I would like to thank Professor Simon Griffith for not only encouraging me to apply to the program, but for taking me on as a student in his lab. The support, guidance, and patience that Professor Griffith bestowed upon me every step of the way throughout this project has been invaluable. His supervision lead me through the completion of this thesis and is necessary component of success in the field of science, especially as beginner scientist like myself facing new experiences daily.

Next, I would like to thank Dr Riccardo Ton for his mentorship over the past 2 years, and contributions to my mist netting, bird banding, and blood sampling skills, all of which have been vital to this project.

I also thank Dr Lori Hurley, whose assistance and expertise during the fieldwork portion of the study provided invaluable, and for dedicating a month of early mornings to join me catching birds in the harsh winter of Broken Hill.

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This project would not have been possible without the combination of attributes that every person I have mentioned brought to the project in order to support me and my learning.

Candidate's statement about the impact of COVID-19 changes on the thesis

Dear Examiner,

Many of our HDR candidates have had to make changes to their research due to the impact of COVID-19. Below you will find a statement from the candidate, approved by their Supervisory Panel, that indicates how their original research plan has been affected by COVID-19 restrictions. Relevant ongoing restrictions in place caused by COVID-19 will also be detailed by the candidate.

Candidate's Statement

I faced COVID related difficulties throughout the project including a 4-month lockdown that restricted and denied access into the laboratory to process samples, and limited the availability of supplies needed to complete the project. This affected my ability to access supplies needed for the project, to perform essential tasks of the project and to do so within the timeframe of the Macquarie MRes program.

I made adjustments were necessary within my abilities due to the severity of COVID-19 restrictions and mentioned these changes in the methodology. I received an extension from Macquarie University of just under three months, moving the submission date from October 25th 2021 to the current due date of February 18th 2022, to account for these set-backs. This extra time allowed me to access the lab to carryout the heterophil/lymphocyte ratio experiments, and the reticulocyte percentage experiments, and also allowed me time to analyse the statistics related to these experiments and write up the details in my manuscript.

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Abstract

Lead is a heavy metal that is often found in high concentrations as a contaminant of urban and industrial environments. Physiological effects of lead on organisms are found to cause negative impacts across neurological, renal, immune, reproductive, haematopoietic, and endocrine systems. The aims of the project were to determine the physiological effects of blood lead levels in a population of house sparrows in the mining town of Broken Hill, NSW, Australia. We took morphological measurements of the sparrows, and collected blood to determine blood lead levels, haemoglobin concentrations and packed cell volume, heterophil/lymphocyte ratios, and reticulocyte percentages. We report blood lead levels >50µg/dL in over 27%, and >100µg/dL in almost 9% of sparrows sampled, indicating significant lead exposure in the population due to environmental contamination (Franson and Pain, 2011). We found a significant negative relationship between haemoglobin and blood lead levels ($P = 0.003$), where individuals with higher blood lead levels had lower haemoglobin levels by around 6%. Unexpectedly, there were no relationships between lead and body condition index, heterophil/lymphocyte ratios or reticulocyte ratios, suggesting that other effects on physiology were limited. This study provides important information on how a bird population is coping with high levels of lead exposure from ongoing environmental pollution.

Introduction

The heavy metal lead (Pb) is commonly found in both natural and highly contaminated environments (Burger and Gochfeld, 2000, Vallverdu-Coll et al., 2015, Sriram et al., 2018). Lead contaminates air, soil, and water and accumulates over time within the ecosystem. Major sources of lead in contaminated environments are from past and current mining, smelting, and refining activities, connected to the extraction industry and through the use of lead-based products such as leaded petroleum (historically), paint, fishing tackle and ammunition (Jenni et al., 2015, Arnemo et al., 2016, Binkowski et al., 2016, Ecke et al., 2017, Grade et al., 2018). The elimination of some of these sources, in addition to stricter regulations on industrial processes and manufacturing of lead-based products, have reduced the introduction of lead into the environment (Scheuhammer et al., 2003). However, historical contamination is highly stable in the soil/ dust and the ongoing effects of lead in contaminated ecosystems remains a problem for people and other organisms (Assi et al., 2016).

The direct deleterious effects of lead on animals have been found in almost every physiological system (reviewed in: (Burger and Gochfeld, 2000, Pain et al., 2019). For example, animals with lead poisoning have been found to have poor renal function and a higher chance of kidney disease (Govind and Madhuri, 2014, Kumar et al., 2020); compromised neurological development leading to behavioural issues, such as increased aggression, learning disabilities, and reduced intelligence (Carpenter, 2001, Mason et al., 2014); various effects on the haematopoietic system including decreases in haemoglobin levels often leading to anaemia, decreased haematocrit levels, changes in the heterophil/lymphocyte ratios, and decreased ALAD enzyme activity (Blus et al., 1999, Franson and Pain, 2011, Van der merwa et al., 2011, Berny et al., 2015); immunosuppression, which can increase the risk of secondary health effects on species (Eeva et al., 2005, Vallverdu- Coll et al., 2019) higher corticosterone levels, and metabolic disease (Eeva et al., 2014, Duan et al., 2020). Reproduction is also quite commonly affected, and lead contaminated birds are found to produce thinner eggshells (Eeva and Lehikoinen, 2015); have a decreased clutch and egg size (Burger, 1995, Berglund et al., 2010); increased nestling mortality (Berglund et al., 2010); and changes to sperm quality and motility (Vallverdu-Coll et al., 2016). The effects of lead are therefore widespread across different physiological systems and through effects on health, reproduction, and mortality, are likely to play a potentially important role in evolutionary selection in contaminated environments.

Whilst most vertebrate taxa have been the focus of work on lead contamination, birds have been particularly well studied, with many studies having characterised cases of lead contamination

in avian populations in the environment. Studies in recreational hunting areas have shown an historically high rate of lead poisoning/toxicity in avian species, with many documented cases of mortality, or birds presenting with chronic lead exposure signs that require medical intervention (Franson and Pain, 2011, Pain et al., 2019). This is due to the ingestion of lead-based pellets or directly from the environment (Mateo, 2009, Newth et al., 2016, Ferreyra et al., 2015, Herring et al., 2020); or by scavenger or predatory through the ingestion of prey species that have been previously hunted with lead-based pellets (reviewed in: (Pain et al., 2019); or via the ingestion of lead-based fishing sinkers by aquatic birds (Scheuhammer and Norris, 1996, Franson et al., 2003, Haig et al., 2014). A well-documented example of avian lead poisoning is observed in the endangered californian condor (*Gymnogyps californianus)*, which has been significantly adversely affected with high levels of mortality (Finkelstein et al., 2012). Despite conservation effects focused on this species, 30% of blood samples collected in an annual survey detected significant levels of lead poisoning at a level known to cause subclinical effects on bird's health and that required intervention to minimise mortality (Finkelstein et al., 2012).

Whilst the avian lead poisoning literature is dominated by species affected by lead shot and lead sinkers, another common pathway to lead poisoning is through exposure via dust bathing and the contaminated food chain in anthropogenically polluted environments (Labare et al., 2004, Gasparik et al., 2012, Godwin et al., 2016). Urban environments are often contaminated with micro particles of lead due to the historic use of widespread lead-based products, such as paint and lead-based petrol. An indication of the scale of the problem was revealed by Roux and Marra (2007) who demonstrated that soil lead concentrations remained significantly higher in urban sites (n = 32) compared to rural sites (n = 21), several decades after lead-based petrol and paint had been banned. The study also found significantly higher blood lead concentrations in six out of seven passerine species surveyed in urban environments, compared to their counterparts in rural settings (Roux and Marra, 2007). These findings are consistent with several other similar studies that have found elevated blood lead levels in urban populations of birds (Hutton and Goodman, 1980Cai and Calisi, 2016, McClelland et al., 2019) other animals such as pet dogs (Balagangatharathilagar et al., 2006, Langlois et al., 2017), and people (Lanphear et al., 1996, de Freitas et al., 2007).

Whilst urban environments are found to generally provide a lead contaminated environment as a result of lead-based petrol and paint (reviewed in: (Levin et al., 2021)), some towns are far worse than others due to elevated lead emissions as a result of industrial and extraction activities. Smelting and mining environments typically have really high levels of environmental contamination, with higher levels of exposure seen in their resident avian populations. For example, Chapa-Vargas et al. (2010) collected 112 blood lead concentrations from 24 bird species sampled

across four polluted sites in a heavily mined region, and one control unpolluted site. Blood lead concentrations were reported in the 1-50.6µg/dL range with significantly higher blood lead concentrations seen in those species sampled from the polluted industrial sites (n = 4) in comparison to those in the control site. Similarly, a single species study of canada geese (*Branta canadensis)* reported that elevated tissue lead concentrations were consistently found in individuals sampled across multiple contaminated mining sites (n = 4) when compared to a non-contaminated reference site, and further linked the lead concentrations to consequential adverse health effects (Van der merwa et al., 2011). Other similar studies document elevated tissue and/or blood lead concentrations due to mining and smelting activities in tundra swans (*Cygnus columbianus)* (, Blus et al., 1991, Blus et al., 1999), northern cardinals (*Cardinalis cardinalis)* and american robins (*Turdus migratorius)* (Beyer et al., 2013), and several species of waterfowl (Beyer et al., 2004)

For many of the birds living in these heavily contaminated mining and smelting areas, the exposure and contamination is chronic, and many studies have examined such populations in an effort to understand the physiological effects of lead exposure on birds. For example, Memishi et al. (2020) found that in a population of feral pigeons (*Columba livia domestica)* in a contaminated smelting environment the average level of blood lead was 7.1µg/dL, and these birds had higher levels of oxidative stress than a similar number of counterparts from an uncontaminated control site. In a population of tree sparrows (*Passer montanus)* in a long-term heavy metal polluted mining site there were smaller clutch sizes and lower fledgling success compared to control sites (Ding et al., 2020). Similar comparative studies documenting lead exposure of birds in mining/smelting sites when compared with non-contaminated control populations have found a range of deleterious effects in a number of bird species (Blus et al., 1993, Blus et al., 1999, Berglund et al., 2010).

When birds are exposed to lead it is absorbed throughout the body, with the highest concentrations typically found in bone, followed by kidney, liver, blood, and feathers in descending order (Pattee et al., 2006, Franson and Pain, 2011, Beyer et al., 2013, Haig et al., 2014). The concentration of lead across different tissues, is due to the way lead is absorbed post-exposure, and the retention capacity of the different tissues and organs. Upon initial exposure, through inhalation or ingestion, some lead stays in the bloodstream whilst the rest is deposited rapidly into various tissues and organs throughout the body, and growing feathers (Fisher et al., 2006, Franson and Pain, 2011, Jenni et al., 2015). Lead in bone is suggested to have the highest concentration due to it being fixed relatively easily (it readily replaces calcium in the matrix, (Mason et al., 2014), loss is slow, and it accumulates throughout the bird's lifetime, thus concentrations can increase with age (Franson and Pain, 2011). Blood lead levels therefore provide good insight into current environmental lead contamination levels as this represents the most recent lead exposure, with a

half-life of approximately two weeks suggested in birds (Pain et al., 2019), and blood sampling therefore provides a non-destructive way of monitoring lead exposure levels in wildlife (Berglund, 2018).

The concentration of lead found in blood, and the tissues, and organs of birds have been well documented and studied in the context of both post-mortem analysis of birds that have succumbed to poisoning, and in intact birds living in contaminated environments. Franson and Pain (2011) collated and interpreted lead tissue concentration data from birds in three families; *Anseriformes, Falconiformes, and Accipitriformes,* whose species have commonly been exposed to lead shot and fishing weights. They proposed three categories of lead poisoning with regards to the level of blood lead: sub-clinical poisoning ranging from 20-50 µg/dL; clinical poisoning from 51-100 µg/dL; severe clinical poisoning at >100 µg/dL on account of the effects that they summarised from the broader literature (Franson and Pain, 2011). This categorisation has since been used as a comparison to findings when investigating lead concentrations throughout the more recent literature (Wiemeyer et al., 2017, Smits and Naidoo, 2018, Lam et al., 2020, Monclús et al., 2020). To further place these thresholds (Franson and Pain, 2011) for birds into broader context, we can consider them against the deleterious effects of lead on health that have been intensively studied in a human medicine context where it is believed that blood lead levels as low as 10µg/dL cause negative health effects (Patrick, 2006), followed by a later suggestion that even levels <10µg/dL still possess great potential to adversely affect human health, particularly in young children (Jusko et al., 2008).

Although studies have adopted Franson and Pain (2011) proposed 20µg/dL to be the standard threshold for non-lethal lead toxicosis in comparison to their findings (Wiemeyer et al., 2017, Ecke et al., 2017, Smits and Naidoo, 2018, Lam et al., 2020, Monclús et al., 2020), there is still uncertainty about a lethal threshold blood lead concentration in avian species, and adverse effects at blood lead concentrations <5µg/dL have also been reported (Pain et al., 2019). The blood lead level of >100µg/dL has been generally accepted as the point at which lethal lead toxicosis will occur (Franson and Pain, 2011). One of the highest blood lead concentrations was recorded in an individual griffon vulture (*Gypus fulvus)* at 1384.44µg/dL, due to the ingestion of lead pellets, who died within 24 hours despite administered supportive treatment (Cairneiro et al., 2016). Table 1 summarises blood lead data from avian species reported in the literature, along with the source of the exposure, demonstrating just how variable the levels can be in birds. Another issue highlighted in Table 1 is the variation in the way which blood lead levels are reported. Even for all these estimates of blood lead, a number of different units are used in the original study, including mg/kg, µg/g, and parts per million or parts per billion. Whilst these can be converted into a common unit, one issue that is less easy to deal with is that some of these blood assays were based on the wet

mass of blood, whilst others were based on the dry mass of blood (see Table 1). A conversion can be applied to convert estimates based on dry mass by using the approximate proportion of water in avian blood (e.g. Hansen et al. 2011), and where appropriate in Table 1 I have converted so that studies can be directly compared.

The thresholds that were established by Franson and Pain (2011) certainly provide a useful guide to the likely clinical outcomes of different levels of contamination. However, there remains uncertainty about the extent to which species or populations will vary in their capacity to withstand the toxic effects of lead at a given level of lead environmental exposure and contamination. In a recent study of 11 house sparrow populations in Australia, Andrew et al. (2019) reported evidence for parallel gene frequency changes in two lead mining towns (Mt Isa, Queensland, and Broken Hill, New South Wales) that differed from those seen in populations in nine other similarly sized non-mining towns. As these changes were independent and the populations were founded by sparrows from quite distinct source populations, they were interpreted as evidence of adaptive selection to a contaminated environment (Andrew et al., 2019). This idea was supported by the fact that many of the specific loci identified were linked to about a dozen genes identified with various roles in metal transport and as being expressed in model species such as the rat (*Rattus norvegicus)* that have been exposed to lead (Andrew et al., 2019). The aim of the current work is to determine the physiological effects of low-level lead exposure in the population at one of these two locations - Broken Hill, in Western New South Wales.

Broken Hill was founded as a mining town over one of the world's largest lead-zinc-silver ore bodies over 130 years ago and has been the focus of continuous mining activity ever since (Taylor et al., 2014). The level of contamination of soil and surface dust has been characterised across the whole town with soil lead (mean: 2,450 mg/kg) and zinc (mean: 3,710 mg/kg) being the most elevated (Taylor et al., 2014). Additionally, when compared to other Australian mining towns with histories of heavy metal contamination, studies have shown Broken Hill to exhibit extremely high levels of lead still present in surface dust samples (Boreland et al., 2002, Taylor et al., 2013). As well as characterising the level of lead in the blood of the Broken Hill House sparrow population, we will investigate a number of physiological parameters that can be used to assess the harmful effects of lead poisoning in this population. The clinical effects of lead exposure on avian physiology have been identified in a number of previous studies (reviewed in: Franson and Pain, 2011, Pain et al., 2019) and a number of these are relatively amenable to study in a non-destructive way allowing them to be employed on a relatively large number of individuals, as described below.

Table 1. Examples of average blood lead levels, sample size, and lead sources of avian studies on lead exposure. Where appropriate conversions from units into µg/dL were done by converting dry weight values into wet weight (dividing by 5.46) and then into volume (dividing by 1.05: estimated blood density factor), before converting into µg/dL units (Hansen et al., 2011). [d]: indicates the individual died and explains such high blood lead levels. [*]: indicates no reference to wet or dry weight was given in the paper, so wet weight was assumed.

Species	Latin name	Lead exposure	Average blood lead reported	Dry (dw) or wet (ww) weight	Blood lead µg/dL (converted)	N	Reference
Golden eagle	*Aquila chrysaetos*	Environmental	0.015µg/g	ww	1.43	397	*Herring et al. 2018*
Bearded vulture	*Gypus barbatus*	Environmental	<3µg/dL	ww	<3	5	*Smits & Naidoo 2018*
Wedge-tailed eagle	*Aquila audax fleayi*	Environmental	3.08µg/dL	ww	3.08	24	*Pay et al. 2021*
White-backed vulture	*Gypus africanus*	Environmental	5.27µg/dL	ww	5.27	11	*Smits & Naidoo 2018*
Golden eagle	*Aquila chrysaetos*	Environmental	0.1mg/L	ww	10	178	*Langner et al. 2015*
White-backed vulture	*Gypus. africanus*	Environmental	10.6µg/dL	ww	10.6	477	*Smits & Naidoo 2018*
Griffon vulture	*Gypus fulvus*	Environmental	15.32µg/dL	ww	15.32	36	*Espin et al. 2014*
White-backed vulture	*Gypus africanus*	Environmental	16.27µg/dL	ww	16.27	90	*Smits & Naidoo 2018*
Cape vulture	*Gypus coprotheres*	Environmental	18.95µg/dL	ww	18.95	49	*Smits & Naidoo 2018*
Black kite	*Milvus migrans*	Environmental	19.43µg/dL	ww	19.43	31	*Carneiro et al. 2018*
Griffon vulture	*Gypus fulvus*	Environmental	41.4µg/dL	ww	41.4	30	*Espin et al. 2014*
Griffon vulture	*Gypus fulvus*	Environmental	43.07µg/dL	ww	43.07	23	*Garcia Fernandez et al.*
Golden eagle	*Aquila chrysaetos*	Environmental	56µg/dL	ww	56	1	*Madry et al. 2015*
Griffon vulture	*Gypus fulvus*	Environmental	821ng/ml	ww	82.1	15	*Vallverdu-coll et al. 2016*
Griffon vulture	*Gypus fulvus*	Environmental	920ng/ml	ww	92	15	*Vallverdu-coll et al. 2016*
Golden eagle	*Aquila chrysaetos*	Environmental	108µg/dL	ww	108	1	*Madry et al. 2015*
Griffon vulture	*Gypus fulvus*	Environmental	805ug/dL*	ww	805[d]	1	*Horowitz et al. 2014*
Griffon vulture	*Gypus fulvus*	Environmental	968µg/dL	ww	968[d]	1	*Carneiro et al. 2016*
Griffon vulture	*Gypus fulvus*	Environmental	1384.44µg/dL	ww	1384.44[d]	1	*Carneiro et al. 2016*
Common eider duck	*Somateria mollissima*	Environmental	0.009µg/g	ww	0.86	190	*Provencher et al. 2016*

Black-bellied whistling duck	*Dendrocygna autumnalis*	Environmental	0.19mg/kg	dw	3.31	17	*Ferreyra et al. 2015*
White-faced tree duck	*Dendrocygna viduata*	Environmental	0.21mg/kg	dw	3.66	67	*Ferreyra et al. 2015*
Whistling duck	*Dendrocygna bicolor*	Environmental	0.31mg/kg	dw	5.68	1	*Ferreyra et al. 2015*
Mallard	*Anas platyrhynchos*	Experimental	11.4µg/dL	ww	11.4	48	*Mateo et al. 2003*
Tundra swan	*Cygnus columbianus*	Environmental	<0.2ug/ml	ww	<20	558	*Ely & Franson 2014*
Mute swan	*Cygnus olor*	Environmental	0.239µg/g	ww	22.76	45	*Meissner et al. 2020*
Whooper swan	*Cygnus cygnus*	Environmental	23.5µg/dL	ww	23.5	260	*Newth et al. 2016*
Tundra swan	*Cygnus columbianus*	Environmental	0.2-0.5ug/ml (range)	ww	20-50	5	*Ely & Franson 2014*
Mallard	*Anas platyrhynchos*	Environmental	1-10µg/g (range)	dw	17.44-174.43	70	*Binkowski et al. 2013*
Cattle egret	*Bubulcus ibis*	Environmental	1.93 µg/g	ww	183.81	10	*Kanwal et al. 2020*
Pond heron	*Ardeola grayii*	Environmental	4.02 µg/g	ww	382.86	10	*Kanwal et al. 2020*
Northern cardinal	*Cardinalis cardinalis*	Environmental	2.50mg/kg	dw	43.61	15	*Beyer et al. 2013*
Andean condor	*Vultur gryphus*	Environmental	15.47µg/dL	ww	15.47	76	*Weimeyer et al. 2017*
White stork	*Ciconia ciconia*	Environmental	103µg/L	ww	10.3	22	*Casa-resino et al. 2015*
White stork	*Ciconia ciconia*	Environmental	44.4ug/L	ww	44.4	35	*Baos et al. 2006*
White stork	*Ciconia ciconia*	Environmental	2.5mg/L	dw	45.79	29	*Kurhalyuk et al. 2009*
White stork	*Ciconia ciconia*	Environmental	7.5mg/L	dw	137.36	28	*Kurhalyuk et al. 2009*
Feral pigeon	*Colombus livia*	Experimental	50.95ppb	ww	5.1	96	*Chatelain et al. 2016*
Feral pigeon	*Colombus livia*	Environmental	17.1µg/dL	ww	17.1	20	*Memishi et al. 2020*
Feral pigeon	*Colombus livia*	Environmental	19.96µg/dL	ww	19.96	250	*Cai & Calisi 2016*
Feral pigeon	*Colombus livia*	Environmental	22.71µg/dL	ww	22.71	63	*Cai & Calisi 2016*
Feral pigeon	*Colombus livia*	Environmental	23.12µg/dL	ww	23.12	39	*Cai & Calisi 2016*
Common loon	*Gavia immer*	Environmental	1.95ppm	ww	195	4	*Franson et al. 2003*
Common loon	*Gavia immer*	Environmental	5.13ppm	ww	513d	2	*Franson et al. 2003*
Northern mockingbird	*Mimis polyglottus*	Environmental	10µg/dL	ww	10	26	*McClelland et al. 2019*

Pied flycatcher	*Ficedula hypoleuca*	Environmental	0.29µg/g	ww	27.6	22	*Berglund et al. 2010*
Pied flycatcher	*Ficedula hypoleuca*	Environmental	0.42µg/g	ww	44.1	27	*Berglund et al. 2010*
Pied flycatcher	*Ficedula hypoleuca*	Environmental	0.78ppm	ww	78	75	*Berglund AMM 2018*
Kaka	*Nestoridae meridionalis*	Environmental	10.8µg/dL	ww	10.8	37	*Sririam et al. 2018*
Northern bobwhite quail	*Colinus virginianus*	Experimental	41.31µg/dL	ww	41.31	48	*Kerr et al. 2010*
Great tit	*Parus major*	Environmental	1.28µg/g	dw	22.33	123	*Bauerova et al. 2020*
Great tit	*Parus major*	Environmental	1.43µg/g	dw	24.94	34	*Bauerova et al. 2020*
Great tit	*Parus major*	Environmental	1.44µg/g	dw	25.12	114	*Bauerova et al. 2020*
Great tit	*Parus major*	Environmental	1.47µg/g	dw	25.64	67	*Bauerova et al. 2020*
Great tit	*Parus major*	Environmental	1.83µg/g	dw	31.92	12	*Bauerova et al. 2020*
Great tit	*Parus major*	Environmental	2.10µg/g	dw	36.63	73	*Bauerova et al. 2020*
Great tit	*Parus major*	Environmental	4.30µg/g	dw	75	3	*Bauerova et al. 2020*
Great tit	*Parus major*	Environmental	7.67µg/g	dw	133.79	5	*Bauerova et al. 2017*
Song sparrow	*Melospiza melodia*	Environmental	1.55mg/kg	dw	27.04	15	*Hansen et al. 2011*
Brown pelican	*Pelecanus occidentalis*	Environmental	2.421ppm	ww	242.1	7	*Franson et al. 2003*
Black-necked stilt	*Himantopus mexicanus*	Environmental	27.5µg/dL (median)	ww	27.5	152	*Riecke et al. 2015*
Eagle owl	*Bubo bubo*	Environmental	2.44ng/mL	ww	0.24	14	*Sanchez-virosta et al. 2020*
Eagle owl	*Bubo bubo*	Environmental	77.9ng/ml	ww	7.79	23	*Sanchez-virosta et al. 2020*
Spotted owlet	*Athene brama*	Environmental	5.24µg/g	ww	499.05	6	*Kanwal et al. 2020*
Swainson thrush	*Catharus ustulatus*	Environmental	0.90mg/kg	dw	15.7	6	*Hansen et al. 2011*
American robin	*Turdus migratorius*	Environmental	3.64mg/kg	dw	63.49	6	*Hansen et al. 2011*
American robin	*Turdus migratorius*	Environmental	7.36mg/kg	dw	128.38	6	*Beyer et al. 2013*

Haemoglobin levels

Haemoglobin is an iron-rich protein found in erythrocytes whose function is to transport oxygen throughout the body. The total haemoglobin concentration is said to provide an accurate estimate of an avian individual's ability to satisfy its physiological oxygen demands (Minias, 2015). Low levels of haemoglobin indicate that an individual has regenerative or non-regenerative anaemia (Minias, 2015), which will reduce oxygen transport to cells throughout the body and can be classified as mild through to severe (Jones, 2015). In non-regenerative anaemia no bone marrow response is stimulated thus no increase in the amount of immature red blood cells (reticulocytes) is found in peripheral blood, whereas increases in reticulocytes are found in individuals with regenerative anaemia (Martinho, 2012). The symptoms and potential negative health effects from avian anaemia are still relatively poorly explored, however anaemia symptoms in most vertebrates include, at a minimum, general weakness and fatigue (Soundarya and Suganthi, 2017).

Several studies have documented the negative effects of lead on haemoglobin concentrations across multiple bird species as lead inhibits haem synthesis resulting in a reduction of new erythrocytes, and an increased destruction of mature erythrocytes, reducing the population of erythrocytes in the blood and consequently the overall concentration of haemoglobin (Martinho, 2012). Most ecological studies use either the total haemoglobin concentration of blood measured directly, or estimated through packed cell volume to reliably determine the blood carrying capacity of oxygen (Minias, 2020). The packed cell volume (PCV) - commonly interchanged with the term hematocrit - is the measure of the volume of red blood cells within the total blood volume when it has been centrifuged down (Fair et al. 2007). It is an additional way to give an indication of the amount of haemoglobin present in an individual, as it has been shown that a reasonable estimate of avian haemoglobin concentration is equal to 0.3 x PCV in multiple species (Velguth et al., 2010). PCV is a commonly utilized tool to indicate the health condition of avian species in the wild and factors including age, sex, reproductive status, and season have been hypothesized to influences PCV changes alongside heavy metal exposure (reviewed in: Fair et al., 2007). Normal PCV levels in captive birds have been established to vary between 35 and 55% of the blood volume, with the rest mostly comprising of plasma (Campbell, 1995).

Previous studies have linked lead exposure in birds to decreases in PCV, often thought to be due to the occurrence of regenerative anaemia (eg. (Fair et al., 2007). A study of tundra swans near a mining and smelting complex found a significant negative relationship between blood lead levels and PCV, with a mean PCV of 47.5% and 0.82µg/g (8.2µg/dL) blood lead in live individuals, and a mean PCV of 36.3% and 3.3ug/g (33.3µg/dL) blood lead in deceased individuals (Blus et al., 1991).

Similarly, studies in trumpeter swans (*Cygnus buccinators)* and canadian geese *(Branta canadensis)* reported significant negative associations between PCV and lead concentration in individuals (Katavolos et al., 2007). Whilst these previous studies were observational, in an experimental study, house sparrows were dosed with varying amounts of Pb (1.3 - >7 µg of Pb/g animal/day) and those exposed to doses above 7.0µg Pb g-1 animal day-1 subsequently showed significant reductions in PCV levels (Cid et al., 2018).

Reticulocytes

Due to the deleterious effects of lead on erythrocytes, they have a higher level of destruction, and the demand for new red blood cells increases. Reticulocytes are immature red blood cells, that are produced in bone marrow and released into the bloodstream to mature (Kalahasthi and Barman, 2016), and are morphologically distinct from mature erythrocytes. The proportion of reticulocytes provides insight into the severity of red blood cell destruction and is often tested alongside haemoglobin concentration and packed cell volume counts in studies of blood lead poisoning (Martinho, 2012, Kalahasthi and Barman, 2016). Whilst little information is available on the life cycle of avian reticulocytes, in mammals the immature cells can be observed in circulating blood for approximately 2 days before they fully mature (Karagulle et al., 2013). When stained and observed with light microscopy they can be seen to have a more rounded shape and nucleus when compared to a mature red blood cell, with a distinct ring encircling the nucleus, and a blue stained cytoplasm (Martinho, 2012, Jones, 2015). From blood smears a count can be made of the number of reticulocytes against mature erythrocytes. In healthy birds, a range of 1-5% reticulocytes has been suggested to be normal (Martinho, 2012), reflecting the natural turnover of erythrocytes which normally last for about 30-40 days in birds (Rodnan et al., 1957). An increase in reticulocyte numbers is suggested to be a likely indication of increased erythrocyte turnover, as a result of increased stress or oxidative damage (Stier et al., 2013). For example, an experimental study orally administered lead doses to 140 young male chickens (*Gallus domesticus*) to determine the effect of the lead on red blood cells and found increases in the percentage of reticulocyte in peripheral blood (Hiraga et al., 2008). Blood lead concentrations peaked between 40-60ppm (dry weight) (697.7-1,046µg/dL) across the lead exposed chicks, with up to 20% of their red blood cells being reticulocytes compared to the control groups (12% reticulocytes) (Hiraga et al., 2008). While the results from this study show lead to effect the number of reticulocytes found in an individual's peripheral blood, the lead dose administered to the chicks was extremely high, and equivalent blood

lead levels are unlikely to be seen in free-living lead contaminated populations, with the exception of individuals that ingest lead-pellets and die (See Table 1).

Heterophils and Lymphocytes

Heterophils and lymphocytes are white blood cells that are involved in immune activity, and the ratio of both in blood smears (H:L ratio) is an increasingly used tool in avian studies of systemic stress (reviewed in: Minias, 2015). A heterophil is described as a round cell with cytoplasmic rod-shaped granules, and is the avian equivalent of the mammalian neutrophil (Maxwell and Robertson, 1998). A lymphocyte is described as a round cell, with an often-centred nucleus, and homogenous cytoplasm (Campbell, 2007). Heterophils and lymphocytes are the most abundant leukocytes observed in birds (Grasman, 2002), and the ratio of these cells in a blood smear is used to identify short- or long-term stress responses in an individual (Maxwell and Robertson, 1998), much like the use of neutrophil:lymphocyte ratios to measure stress responses in human medicine (e.g. Song et al., 2021). Generally, an increase in the H:L ratio represents exposure to short- or long-term stress in an individual (Skwarska, 2019). However, both increases and decreases in the H:L ratio have been reported in lead exposure studies in birds. For example, in a study of great tits in which individuals at the most polluted site with an average blood lead level of 7.67µg/g (133.79 µg/dL), were found to have a significant decrease in H:L ratios when compared to those with elevated feather lead levels which saw significant increases in H:L ratios (Bauerova et al., 2017) suggesting that both increases and decreases in H:L ratios were signs of physiological stress occurring due to lead exposure. A recent experimental study of great tit nestlings found a significant decrease in H:L ratios in individuals with increased blood lead levels with the highest lead dosed group (30µg/Pb per gram of body mass) shown to have the lowest H:L ratios (Markowski et al., 2019). Similar recent experimental studies in house sparrows (Cid et al., 2018), and mute swans (*Cygnus olor)* (Meissner et al., 2020) have also shown H:L ratios to increase significantly in individuals with higher blood lead levels. Whilst it is not clear why both negative and positive relationships have been seen, all studies mentioned above have typically found a significant relationship between lead and H:L ratios, supporting the idea that this is a useful measure of the deleterious effects of lead in birds.

Body condition

Simple morphometric measurements are long-established approach in determining health and estimating body condition in bird species. Body condition is generally classified as an individual's energy reserves in relation to its body size (Hill et al., 2003, Blums et al., 2005). Morphological measurements commonly utilized include body mass, tarsus and wing chord measurements, fat scoring, or a combination of these (reviewed in: Labocha and Hayes, 2011). Body condition indices have been linked to an individual's fitness, survival rates, ability to reproduce, and even behavioural responses. For example, survival rates of female mallards (*Anas platyrhynchos*) were predicted by body condition (Bergan and Smith, 1993), and in a study of three species of duck (tufted duck, *Aythya fuligula*; common pochard, *Aythya ferina*; northern shoveler, *Anas clypeata*) across fifteen years birds classified in better body condition showed the highest rate of survival, compared to those in poor condition (Blums et al., 2005). Multiple other studies have shown links between good body condition and an increase in survival rates in multiple bird species (Dufour et al., 1993, Schmutz, 1993, Hill et al., 2003).

The effects of lead on body condition have been addressed in numerous studies. Franson and Pain (2011) suggested that most avian studies in lead exposed individuals with poor body condition are those of extreme lead exposure cases (i.e.. Blood lead levels >100µg/dL), however, studies since have suggested that even lower levels of lead exposure can inflict significant decreases in body mass and negatively influence body condition of avian species. For example, the significant negative relationship between blood lead levels greater than 44µg/dL and body condition in a population of whooper swans (*Cygnus cygnus*) (Newth et al., 2016), and again in a study by Gillingham et al. (2021) that reported the bioaccumulation of lead in the feathers of population of greater flamingo (*Phoenicopterus* roseus) chicks from polluted sites (max. lead 0.065mg/kg wet weight; equivalent to 6.19µg/dL) having a strong negative effect on body condition.

The aims of the project were to determine the physiological effects of blood lead levels in a population of house sparrows in the mining town of Broken Hill, NSW, Australia. To do this we determined the relationship between blood lead levels and select physiological parameters including haemoglobin levels, heterophil and lymphocyte levels, reticulocyte counts, and body condition indices. We expected to see individuals with higher blood lead levels, particularly those greater than 50µg/dL, to suffer negative physiological effects.

Methods

During the years 2020 and 2021, samples were collected from the population of house sparrows in Broken Hill (2020: n = 218, 2021: n = 208). The birds were captured using mist nets and kept in individual bird bags for a short time until sampling could be conducted. Sampling was undertaken under the Animal Research Authority of the Animal Ethics Committee at Macquarie University (ARA 2020/011-4). In 2020 we sampled as many locations as possible fairly evenly distributed across the whole town (sites N = 43), to characterise the blood lead levels across the whole Broken Hill house sparrow population (Figure 1). Using the 2020 site blood lead level data across the town, we went back in 2021 to 13 sites that were selected to allow us to sample sparrows in specific blood lead ranges for further work focused on health and physiology (Figure 2). As a result of multiple factors including blood sampling volume limitations, equipment access and availability, and time constraints for sample analysis, we often collected a larger sample size for certain physiological parameters than others and often collected more samples than time, or money allowed us to later analyse in the laboratory. A full table of all sampling done for each individual sparrow can be found in the supplementary materials (T1, T2, and T3).

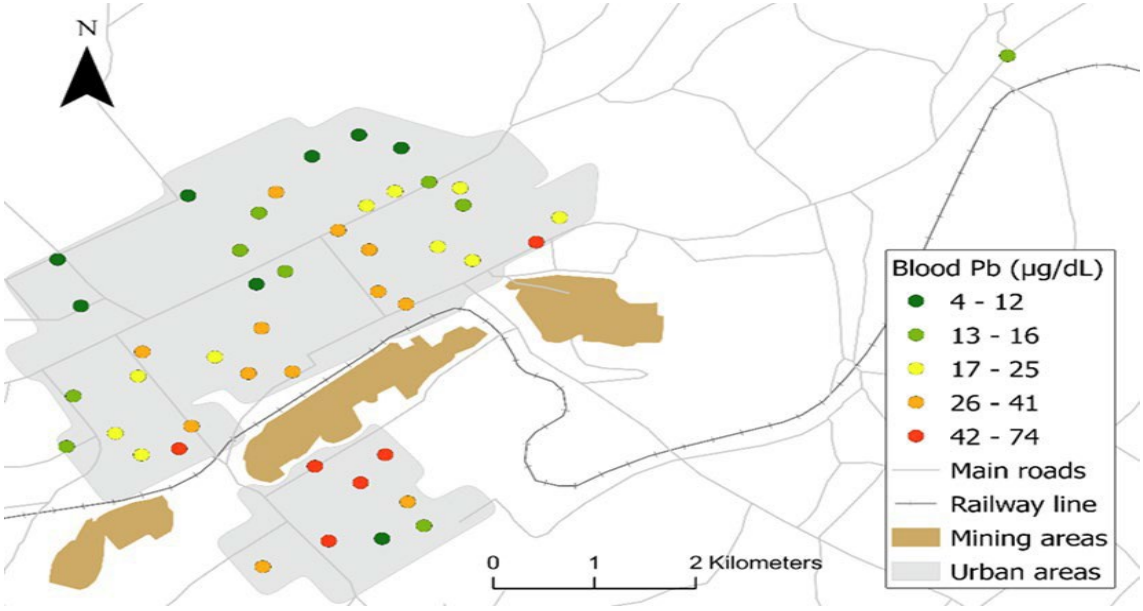

Figure 1. A map showing the 2020 average blood lead levels for each catching site (n = 45), represented by an individual circle. An average of 5 birds per site were tested for blood lead level data (with n = 2 sites having 15 blood lead samples taken) in order to establish an idea of the overall blood lead levels in the sparrow population of Broken Hill. This allowed us to determine the specific areas to catch at for the 2021 data collection. *Map produced and provided for use in this thesis by Max McLennan-Gillings, 2021.

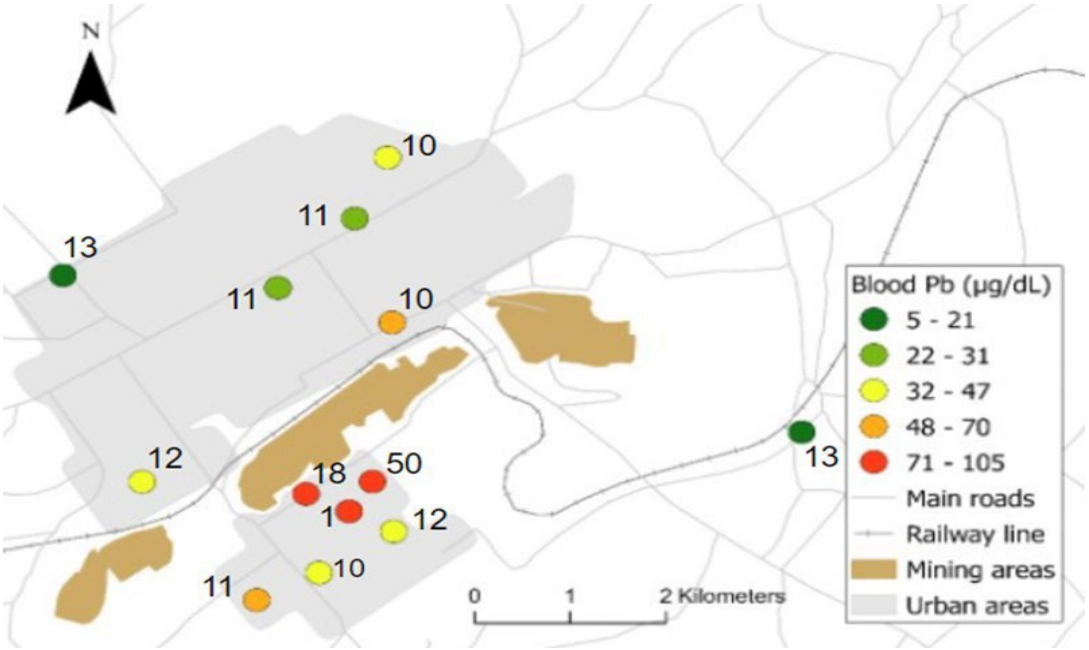

Figure 2. A map showing the 2021 average blood lead levels for each catching site (n = 13), represented by an individual circle. Sites were chosen specifically based off the 2020 blood lead levels so as to get a more biased amount of high-end blood lead level individuals (>70 µg/dL), and low-end blood lead level individuals (<20 µg/dL), as well as a few mid-range blood lead levels individuals (20-70 µg/dL). This was done to show the effects the extreme end lead exposure has on the physiological parameters when compared to background lead exposure based on previous lead exposure effects in avian literature (Franson and Pain, 2011) . Sample numbers indicated on map in bold for each site. *Map produced and provided for use in this thesis by Max McLennan-Gillings, 2021.

Sample collection

At the time of initial capture, each bird was banded with an individually numbered steel band from the Australian Bird and Bat Banding scheme for identification, and in case of future recapture. Morphometric measurements were taken including; mass recorded to the nearest 0.01 grams, and tarsus and flattened wing chord body measurements which were recorded using digital callipers to the nearest 0.01 millimeters. The length of the tarsus was classified as from the tarsal joint to the base pad of the foot. The length of the wing chord was classified as from the carpal joint to the tip of the longest feather, vertically. In one event where the digital callipers failed, no data was recorded, and in a second event of the same circumstances, analogue callipers were available to record measurements to the nearest 0.1mm.

Sexing and approximate ageing of individuals was undertaken where possible. Birds were assigned as either male or female when adult sexually dimorphic plumage was clear (as described in

(Joshi, 2009). If the bird was suspected to be of juvenile age, skull aging techniques were used alongside the state of the plumage moult as juveniles moult into adult plumage (Nero, 1951). Fat scoring of individuals was done visually by assessing the amount of subcutaneous fat in the axillary region of the pectoral area and abdomen (widely accepted avian visual fat scoring method developed by (Helms and Drury, 1960). To assess the amount of fat present, the bird was laid on its back with the neck between the second and third finger of the hand, and feathers were moistened with warm water with the second hand to expose the area. We used six fat score categories, ranging from 0-5 (based on descriptions in (Krementz and Pendleton, 1990).

Blood samples were collected from sparrows by puncturing the brachial vein with a 26-gauge needle and collecting the blood with a 50µl capacity capillary tube. All individuals were blood sampled with at least one sample collected into 95% ethanol for later DNA work (not the subject of this thesis). However, blood was also collected for a number of other assays, depending on the field trip (2020 or 2021), the data requirements at particular sites and logistic constraints at the time of collection. On some days we were particularly busy and were not able to collect all samples from every bird. For the sampling of lead, given the cost of running assays, we only sub-sampled a proportion of the individuals caught in each location. For lead assays we took two different approaches. One sample of around 50µl of blood immediately transferred from the micropipette into a LeadCare Plus® test kit Eppendorf tube containing Hydrochloric acid. The tube was inverted ten times and placed on ice, in a cooler, while in the field. In many cases a second sample was taken for lead and trace metal analysis into an empty 1.5ml Eppendorf which was held on ice and then stored frozen until it could be assayed later in Sydney. In 2021 we also took additional blood samples for physiological assays. A small drop of blood was put into a HemoCue® Hb 201+ photometer (HemoCue AB, Angelholm, Sweden) microcuvette and tested in field. Finally, approximately 5µl was taken dropped onto a clean microscope slide, and used to create a blood smear, using a second slide to create a thin layer of blood (following (Owen, 2011). The slide was allowed to air dry before being stored in a slide box.

Primarily, we relied on the HemoCue® Hb 201+ photometer to provide the haemoglobin concentration for our study. After most of the fieldwork had been carried out, we returned to the field to collect samples from two previous sampled sites, one representing low lead individuals and one representing high lead individuals. During this time, we sampled birds again for haemoglobin concentration (following the same method) and also took a blood drop for the analysis of packed cell volume, with approximately 20 µl collected in a new heparinised microcapillary tube that was sealed with tube sealing clay and stored on ice for later centrifugation. This enabled us to ascertain that our HemoCue measures did indeed reflect the number of erythrocytes present.

After the blood sample was taken and the bird had recovered, the birds were released at the site of capture.

Sample Processing

The primary method of lead quantification was to use a LeadCare Plus® Blood Lead Analyser Model 82-0002 (Magellan Diagnostics, Inc. 2019). Samples were tested within 12 hours of initial collection. Reagent tubes were inverted and left to rest in the upright position for 30s. A LeadCare sensor was inserted into the machine and 30µl of sample was pipetted from the reagent tubes onto the sensor. A blood lead level reading was given by the machine after 180s. Blood lead readings were given in micrograms (µg) of lead per decilitre (dL) of whole blood, with the lowest limit of detection at 1.8µg/dL and the end range limit of detection at 65µg/dL. Samples reported under the minimum detectability (ie. <1.8µg/dL) were given the value 1.7µg/dL (n = 6), and those reported above the maximum detectability (ie. >65µg/dL) were given the value 65.1µg/dL (n = 19) for data analysis.

After the fieldwork had been carried out, we were notified by the manufacturer of consistent underestimation issues with the testing device. Under estimation in LeadCare testing devices has been documented previously in other avian field studies, and we applied methods based on those studies (Craighead and Bedrosian, 2008, Green et al., 2008, Langner et al., 2015, Gonzalez et al., 2019), to account for the issue, using direct measures of lead in the other blood sample we had taken for a subset of individuals. We submitted samples taken from 30 individuals (in 2020), for which we had a paired LeadCare (LC) value, for inductively coupled plasma mass spectrometry (ICP-MS) trace metal analysis conducted by the Australian Government National Measurement Institute (NMI). The NMI returned the amount of Lead, and 14 trace metals, found in the sample in mg/kg and we converted this into a blood concentration using the blood density of 1040 kg/m^3 (from chickens, Medway and Kare, 1959). There is no blood density available for any passerine in the literature and we assumed that, House sparrow blood has a similar density to the chickens. We compared the blood lead data assayed using the ICP-MS and original LeadCare values, using a linear regression model. The linear regression model (y-intercept = 0) was performed under the assumption that at a LeadCare concentration of 0 (independent variable), the ICP-MS concentration would be 0 (dependent variable). We then fitted a linear trend to the relationship between the two variables which gave us a strong positive linear relationship ($r = 0.93$, $p < 0.01$, $n = 30$; Supplementary F2) and the regression equation ($y = 1.8296x$), which we used to correct the values that were taken with the LeadCare assay in both years 2020 (n = 218) and 2021 (n = 182). The

original reported LeadCare blood lead values are provided in the supplementary material (Table 1), along with the corresponding corrected value. Note that this correction does not alter the blood lead differences between individuals, rather provides a more accurate representation of the absolute blood lead concentrations found within the population for comparison to previous studies (shown in Table 1).

An individual's haemoglobin concentration was calculated in the field using the HemoCue® Hb 201+ photometer which displayed the value on the screen approximately 30-60 seconds after inserting the microcuvette with the blood sample. The level of detection ranges from 0 to 256 grams (g) of haemoglobin per litre of whole blood (L). The optronic unit was cleaned regularly using the suppliers cleaning kit and method. To validate this machine as an accurate method of measuring haemoglobin in sparrows, and to help us interpret the results, for a sub-sample of individuals we measured the packed cell volume (PCV). PCV was measured using whole blood samples in heparinised microhematocrit tubes which were stored on ice for up to 2 hours and were then centrifuged in a benchtop centrifuge fitted with a rotor designed to hold microhaematocrit tubes, for 5 minutes at 12,753rpm (SIGMA 1-14 centrifuge). We then measured the plasma and red blood cell fractions using callipers and calculated PCV as a percentage using the equation plasma (RBC/(RBC + Plasma) * 100).

Blood smears were stored at room temperature and in the dark to preserve sample integrity until they were stained approximately 5 weeks after returning from the field. Due to limited time in the laboratory (due to COVID-enforced lockdown), we processed smears from individuals with the highest (n = 23) and lowest (n = 27) blood lead levels in the samples we had collected. We stained the blood smear using a Wright's-Geimsa solution (Point of Care Diagnostics). Slides were placed vertically in a staining rack, and in deionised water for 5 minutes to rehydrate the smears, before being placed in a bath containing the staining solution for 3 minutes, followed a bath with deionised water for 1 minute then rinsed and placed upright in a fume hood to air dry. Water baths were changed every 40 slides stained to ensure proper rinsing. The slides were then examined under an optical microscope (Olympus BX50, Japan) with an Industrial Digital Camera attachment (14MP ½.3" Colour, USB2.0, Aptima CMOS Sensor). For each slide we used a 60x magnification to identify and count different leukocytes to a total cell count of 100 by moving in a consistent way from left to right across the smear, to ensure that cells weren't double-counted. On slides where 100 was difficult to achieve, a minimum total of 50 leukocyte identified was accepted (average: 61, range: 50-108), with monocytes, basophils, and eosinophils excluded from counts. Images were

taken of white blood cells for each sample, to use for later scoring. A heterophil/lymphocyte ratio was calculated for each individual as the number of heterophils/number of lymphocytes.

Reticulocyte counts were performed using a random sub-set of the same images of blood smears used for the white blood cell counts. The reticulocytes and normal erythrocytes were counted using the freely available program DotDotGoose (Version 1.5.1). This software allows a user to mark each cell on an image as being either one or the other, and then provides the total count of each. Due to our Wright-Geimsa staining protocol being suitable for distinguishing both mature and immature erythrocytes, but not the various maturation stages, we identified definite reticulocytes by their larger size, lesser condensed nucleus, and blue-grey stained cytoplasm (Jones, 2015). A minimum total of 1000 cells were counted (average: 1068, range: 1006-1109). The percentage of reticulocytes was calculated as the (number of definite reticulocytes/total red blood cells) × 100.

Statistical analyses

One-way ANOVA analyses were used to compare blood lead levels between adults and juveniles and between males and females, for both 2020 and 2021 data sets. One-way ANOVA was also performed to compare blood lead levels from the sites sampled in 2020, and to compare BCI to fat scores of individuals. A Spearman Correlation analysis was performed on the ICP-MS trace metal concentration data to investigate correlations between the different metals, and corrected LeadCare data for 41 individuals.

All statistical analyses described below were performed using RStudio for Windows (Version 1.4.1717, RStudio, Inc. Boston, MA, US). A linear model (LM) approach was used for all models under the "lme4" package (Bates et al. 2015). The factor 'body condition index' (hereafter referred to as BCI), was created by calculating the residuals of body mass against tarsus length using the dplyr package (Wickham et al. 2021). Separate LM models were used to test whether haemoglobin levels, H/L ratios, and reticulocyte counts were affected by the same fixed effects: lead, sex, BCI, and their two-way interactions. Non-significant interaction terms were removed in a step-wise manner, starting with the largest p-value. For each LM, we calculated marginal R^2 values to determine how much variation was explained by our fixed effects. A p-value <0.05 was considered statistically significant for all models. 95% confidence intervals (CI) were calculated for each step within each model. Model fit was assessed by examining model residuals. Non-significant terms were removed in a step-wise manner, with non-significant interaction terms removed first.

Cubic transformation was performed on haemoglobin data to correct to a normal distribution. Haemoglobin modelling was then performed once including all haemoglobin values and all lead data, and separately without those individuals for which the LeadCare readings were outside of the limit of detection (ie. <1.8µg/dL and >65µg/dL) in order to determine if there was a significant effect of these unknown values on the modelling. Log transformation was performed on the H/L ratio to fit the normal pattern of distribution. Samples were sorted into two groups for comparison, based on recorded blood lead levels, and due to COVID restricting access to the laboratory we prioritised the processing of the samples from the individuals for which we had identified the highest and lowest blood lead levels. Those assigned into the "low" group had blood lead levels ranging between 3.29-18.66µg/dL, and "high" ranging between 90.2-119.11µg/dL. Modelling was performed with all interactions and fixed effects. The full reticulocyte data set was modelled with all interactions and fixed effects. BCI was modelled with only lead and sex fixed effects and their two-way interaction.

Due to concerns about juveniles having functionally different immune system and possible differences in reactions to lead exposure when compared to adults, all modelling was performed with juveniles included (full data set), and again with only adult data (juveniles removed). This was to ensure that juvenile data did not unduly influence the model outcomes, but also to investigate the possible differences between adults and juveniles.

Results

Blood lead

2020: We recorded the blood levels of 218 individuals, comprising 179 adults (102 male, 77 female), and 39 juveniles (unsexed) across 43 sites (Figure 1). We report no significant difference in average blood lead levels between adults and juveniles (P-value: 0.64, Supplementary T4) or between sexes (P-value: 0.12, Supplementary T5). LeadCare reported blood lead levels ranged from ≥1.8 to <65 µg/dL, with an average of 15.2µg/dL. This average includes birds with blood lead readings below (n = 3) and above (n = 2) the LeadCare limit of detection (≥1.8 to <65 µg/dL). We assigned the low samples as 1.7µg/dL (adjusted value = 3.1µg/dL) and the high samples as 65.1µg/dL (adjusted value = 119.11µg/dL) for the purpose of calculating this average value. Adjusted blood lead levels ranged from 3.1 to 119.1µg/dL, with an average of 27.8µg/dL (Figure 3). The overall frequency distribution of the adjusted blood lead levels for the data collected in 2020 sparrows can be seen in Supplementary T3. Of the sampled population 11.5% (n = 25/218) individuals across town had blood lead levels considered sub-lethal (>51µg/dL), and 2% (n = 4/218) had blood lead levels >100µg/dL which are considered lethal (Franson and Pain 2011). We found a significant difference between blood lead averages across all catching sites (One-way ANOVA. $F_{36,179}$: 7.65. P-value: <0.0001. Supplementary T9).

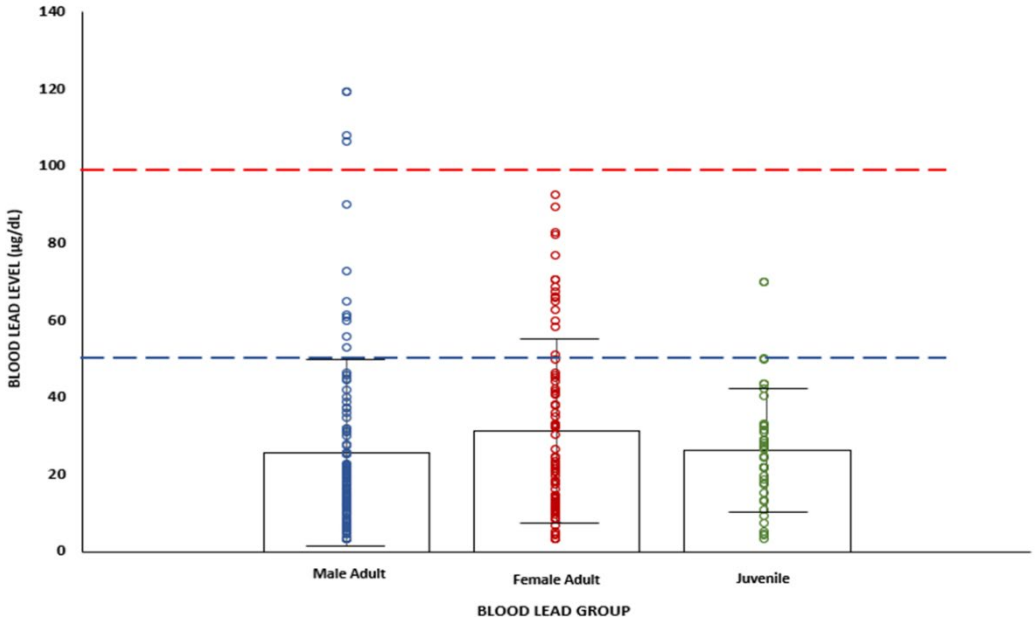

Figure 3. 2020 distribution of sparrow blood lead levels (n = 218). The blue line indicates chronic exposure (>50µg/dL), the red line indicates lethal exposure (>100µg/dL) (Franson and Pain, 2011).

2021: We recorded 182 blood lead levels of birds sampled at 13 sites across town (Figure 2), from 157 adults (79 male, 78 female), and 25 juveniles (10 male, 14 female, 1 unsexed). We found no significant difference in the average blood lead levels of adults and juveniles (P-value: 0.57, Supplementary T6) or between sexes (P-value: 0.27, Supplementary T7 and T8). In the low lead areas, we found a range of 3.11-119.1µg/dL in 91 individuals with an average of 29.4±2.9µg/dL blood lead, and in the high lead areas a range of 18.7-119.1µg/dL in 91 individuals, with an average blood lead level of 77.5±3.2µg/dL. The overall frequency distribution of the adjusted blood lead levels for the data collected in 2021 sparrows can be seen in Supplementary T1. The frequency of the adjusted blood lead levels distribution across the sampled sparrows, in comparison with literature suggested sub-lethal and lethal threshold values, can be seen in Figure 4.

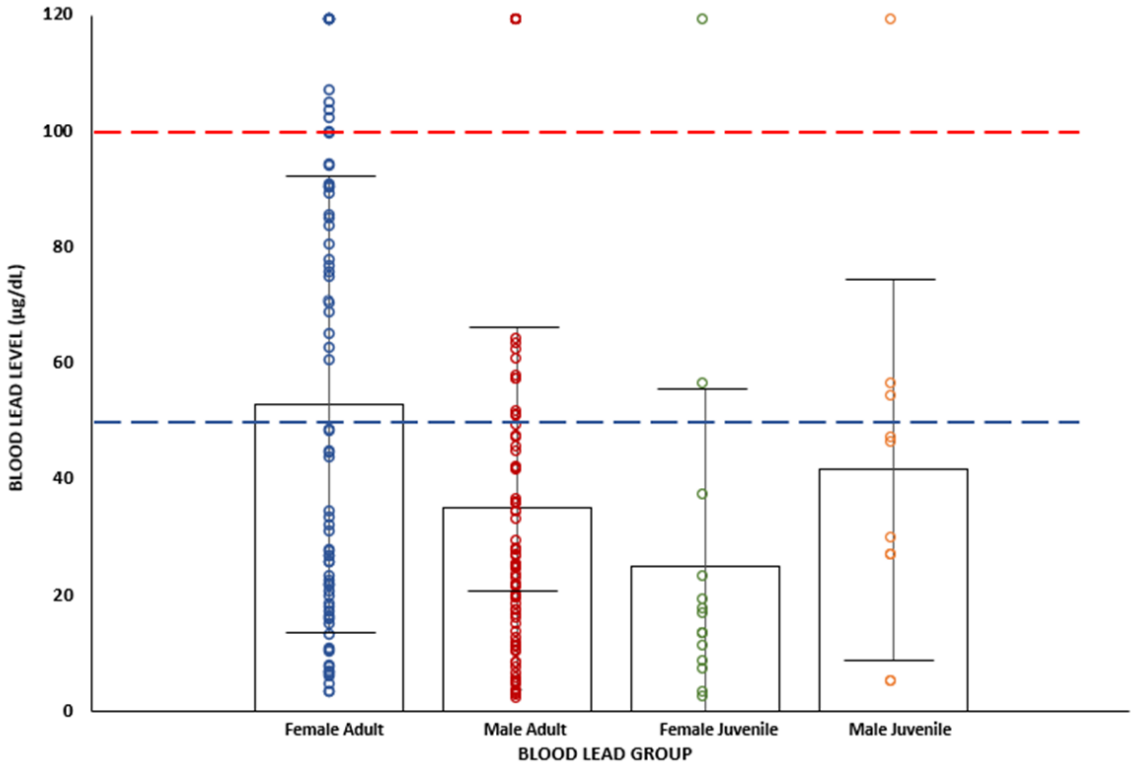

Figure 4. The distribution of sparrow blood lead levels in 2021 (n = 182). We sampled more individuals from sites that we had previously determined to have higher blood lead in 2020, explaining the upward bias of blood lead levels in this sample. The blue line indicates chronic exposure (>50µg/dL), and the red line indicates lethal exposure (>100µg/dL) (Franson and Pain, 2011).

Haematological analyses

Haemoglobin: We recorded the level of haemoglobin in 162 adults (81 males, 81 females), and 25 juveniles (10 males, 14 females, 1 of undetermined sex). In this whole sample, the haemoglobin concentration varied between 106-199g/L, with an average of 164.25g/L (Figure 5). We found a negative relationship between blood lead levels and haemoglobin (R^2: 0.048, P: 0.0032; Table 4). None of the other fixed factors or their two-way interactions were found to have significant effects on haemoglobin levels (Supplementary T10). The average haemoglobin level for birds with low blood lead levels (<20µg/dL; considered background exposure levels by Franson and Pain, 2011) was 5.9% higher than those with high levels (>100 µg/dL; considered lethal by Franson and Pain, 2011). Figure 7 shows the raw haemoglobin data of the low and high blood lead groups, along with the mean. The mean, standard error and sample size for both lead groups can be seen in Table 2. After removing the juvenile data, and repeating the same analysis we also confirmed that haemoglobin was negatively related to the level of blood with lead (Table 3) and no other fixed factors or their two-way interactions (R^2: 0.053, P: 0.0039) (Supplementary T11).

Our measures of haemoglobin concentration with the HemoCue® Hb 201+ photometer, was validated by a linear regression showing the strong positive relationship between the haemoglobin concentration measured with this device, and the packed cell volume measured in a subset of 56 birds (R^2: 0.495, Figure 6).

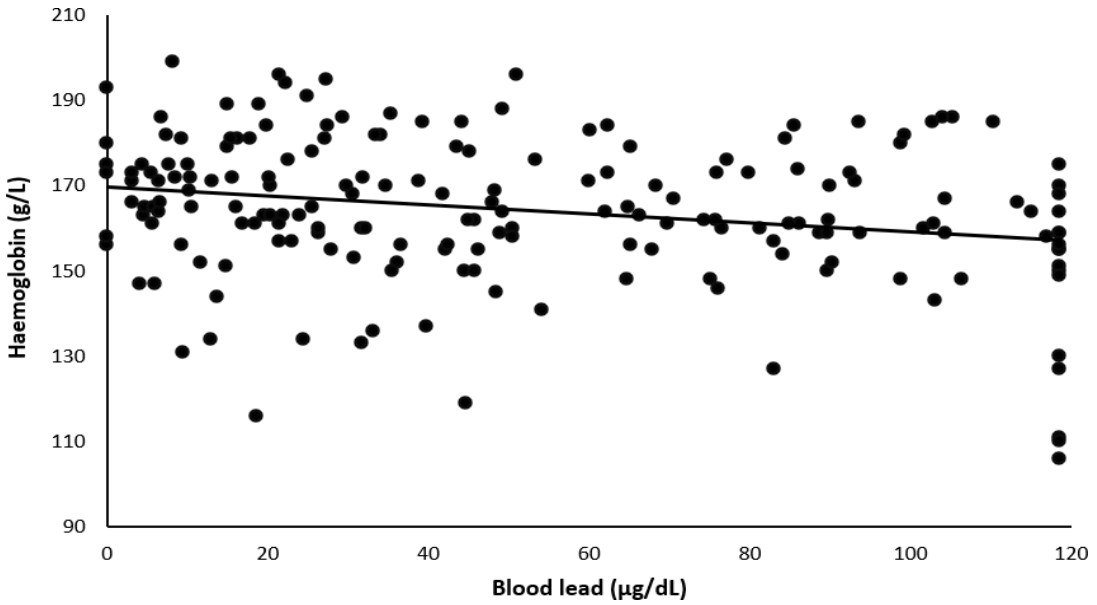

Figure 5. A scatter plot showing the negative relationship between haemoglobin (g/L) and blood lead levels (µg/dL) in House sparrows (n = 187). R^2: 0.048, DF: 179, P-value: 0.003.

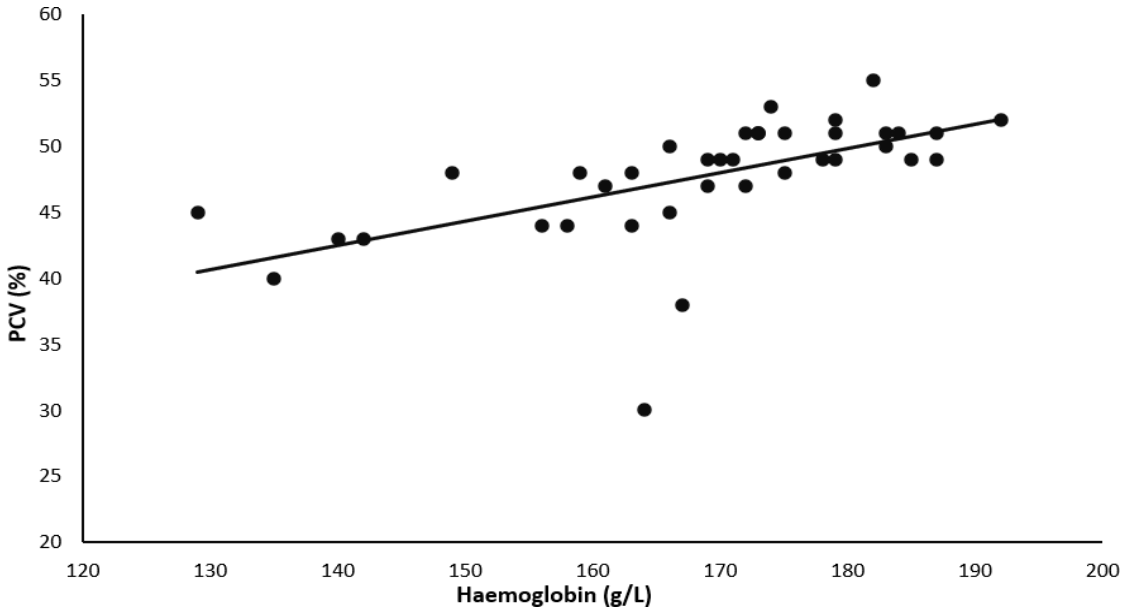

Figure 6. A scatter plot showing the relationship between haemoglobin (g/L) and PCV in House sparrows (N = 55). R^2: 0.334.

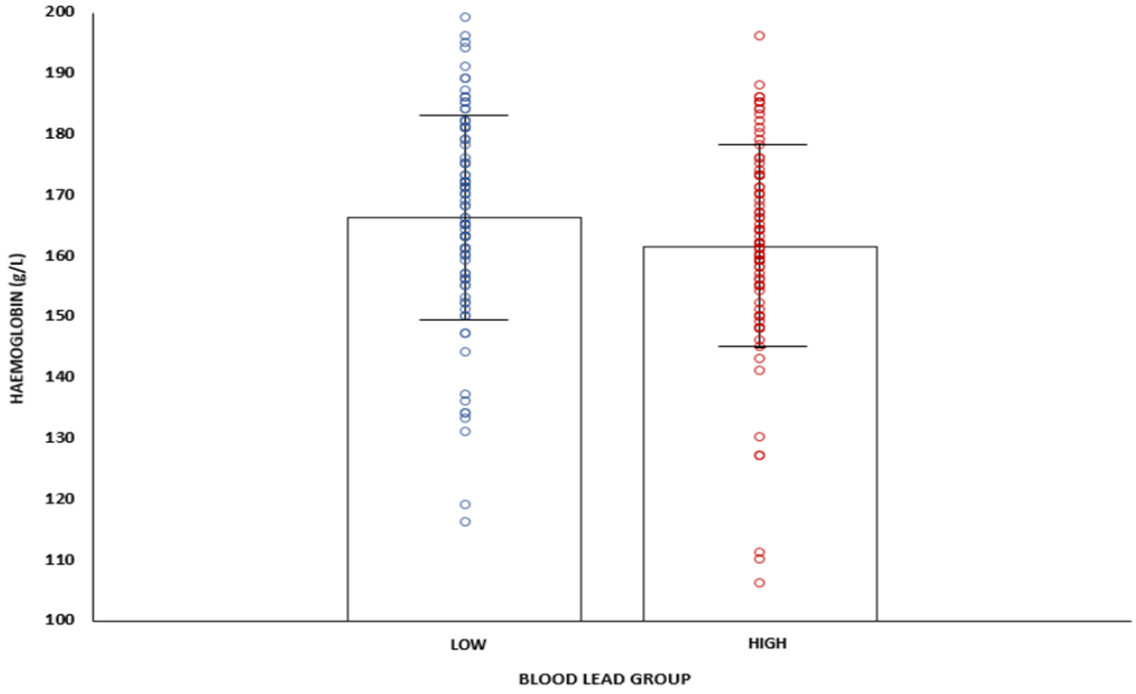

Figure 7. The haemoglobin concentration of sparrows with low or high blood lead levels. Samples were split into two groups based on blood lead levels of the individual. As we had a larger data set for this physiological parameter compared to other, here we split the blood lead groups into three ranges, with the "Low" group indicative of sparrows with blood lead levels <20µg/dL (n = 42; mean = 167±1.73), and the "High" group is indicative of sparrows with blood lead levels >100µg/dL (n = 33; mean = 157±1.77). Individual data points are presented with the blue group representing low blood lead and the red group representing high blood lead, along with their corresponding mean (height of the bar) ± SD.

Table 2. The mean, standard error, and sample size for all the samples collected and analysed including: Haemoglobin, H/L ratio, Reticulocytes, and PCV.

Parameter	*Lead group (µg/dL)*	*Mean ± Standard Error*	*Sample size (n)*
Haemoglobin (g/L)	Low (<20)	167±1.73	42
	High (≥100)	157±1.77	33
H/L Ratio	Low (<19)	1.67±0.24	27
	High (>90)	1.29±0.20	23
Reticulocytes (%)	Low (<20)	2.77±0.33	29
	High (>93)	2.76±0.32	27

Table 3. Linear model final output to examine haemoglobin (n = 187). A significant relationship was seen with lead as an independent factor. Multiple R^2: 0.048. Adjusted R^2: 0.04. F-statistic: 8.96 on 1. Df: 179. P-value: 0.003.

Haemoglobin						
	Estimate	**SE**	**T value**	**P-Value**	**Confidence Intervals**	
					2.5%	97.5%
Intercept	4858154	183969	26.41	<2e-16**	4494965	5221343
Sex	147689	193037	0.77	0.45	-233402	528781
BCI	-77393	62129	-1.25	0.21	-200043	-2002
Lead	-7479	2498	-2.99	**0.003****	-12409	-2549

Heterophil/lymphocyte ratio: White blood cell counts were scored for 50 birds (45 adults: 19 males and 25 females, and 6 juveniles: 3 males and 3 females), the raw data of the individual H:L ratio values can be seen in Figure 8. The mean, standard error and sample size for each group can be seen in Table 2. We ran the H:L ratio model to explore any potential relationships with fixed factors and their two-way interactions. We found a significant positive relationship between H:L ratio and BCI (R^2: 0.814, P: 0.0005; Table 4), with all other covariates insignificant and removed from the final model (Supplementary T12). Individuals with a high body condition had a higher proportion of heterophils to lymphocytes. Blood lead levels were found to have no effect on H:L ratios in an individual. After removing juvenile data, and re-running the models, we again found a

significant relationship with BCI but not with remaining fixed factors or their two-way interactions (Supplementary T13).

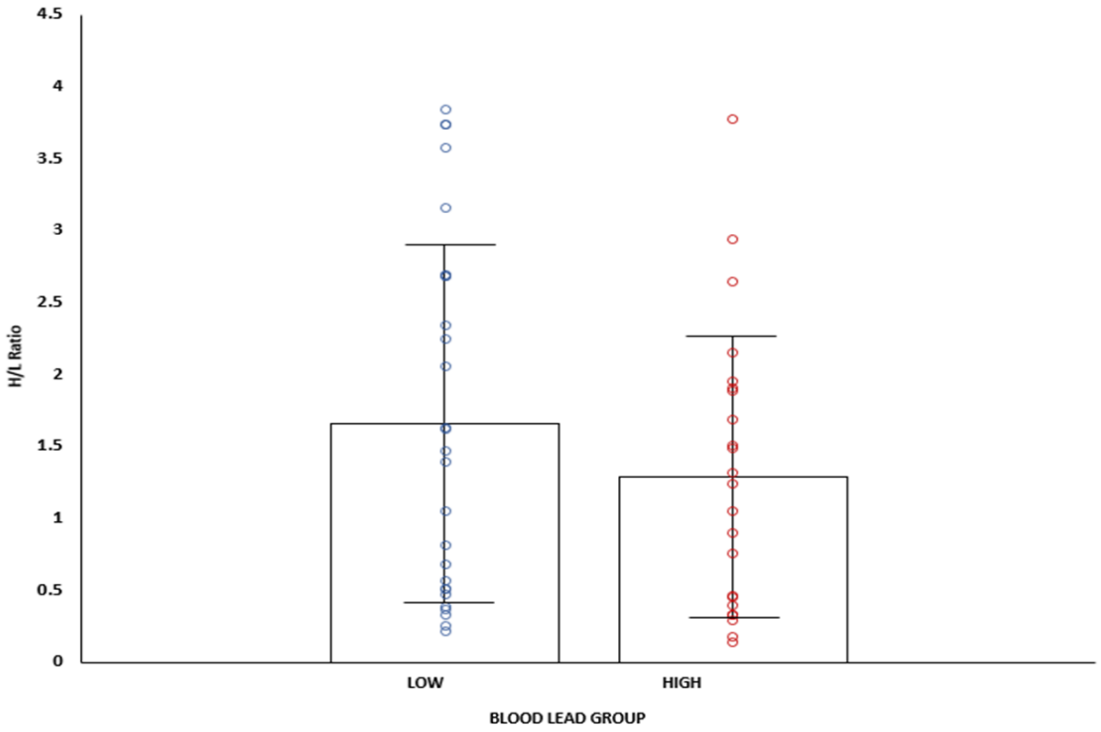

Figure 8. The H:L ratio of sparrows with low or high blood lead levels. Samples were split into two groups based on blood lead levels of the individual. The "Low" group (represented by blue markers) is indicative of sparrows with blood lead levels <19µg/dL (n = 27, mean = 1.67±0.24) and the "High" group (represented by red markers) is indicative of sparrows with blood lead levels >90µg/dL (n = 23, mean = 1.29±0.20). Individual data points are presented, along with their mean (height of the bar) ± SD.

Table 4. Linear model final output to examine heterophil/lymphocyte ratio (n = 50). A significant relationship was seen with body condition index. Multiple R^2: 0.23. Adjusted R^2: 0.22. F-statistic: 13.95 on 1. Df: 47. P-value: 0.00005.

H/L Ratio						
	Estimate	**SE**	**T value**	**P-Value**	**Confidence Intervals**	
					2.5%	97.5%
Intercept	0.03	0.21	0.15	0.88	-0.40	0.46
Lead	0.03	0.24	0.13	0.90	-0.46	0.52
Sex	-0.09	0.24	-0.38	0.71	-0.58	0.40
BCI	0.26	0.07	3.73	**0.0005***	0.12	0.41

Reticulocytes: We made reticulocyte counts for 56 adults (27 males, 29 females), with the raw data and mean of individual counts illustrated in Figure 9. The mean, standard error and sample size for the high and low blood lead groups can be seen in Table 2. The percentage of reticulocytes in an individual's blood was significantly related to an individual's sex, with females having a higher percentage on average than males (R^2: 0.107, P-value: 0.014; Table 5). We found blood lead levels to have no effect on the percentage of reticulocytes in an individual (Supplementary T14).

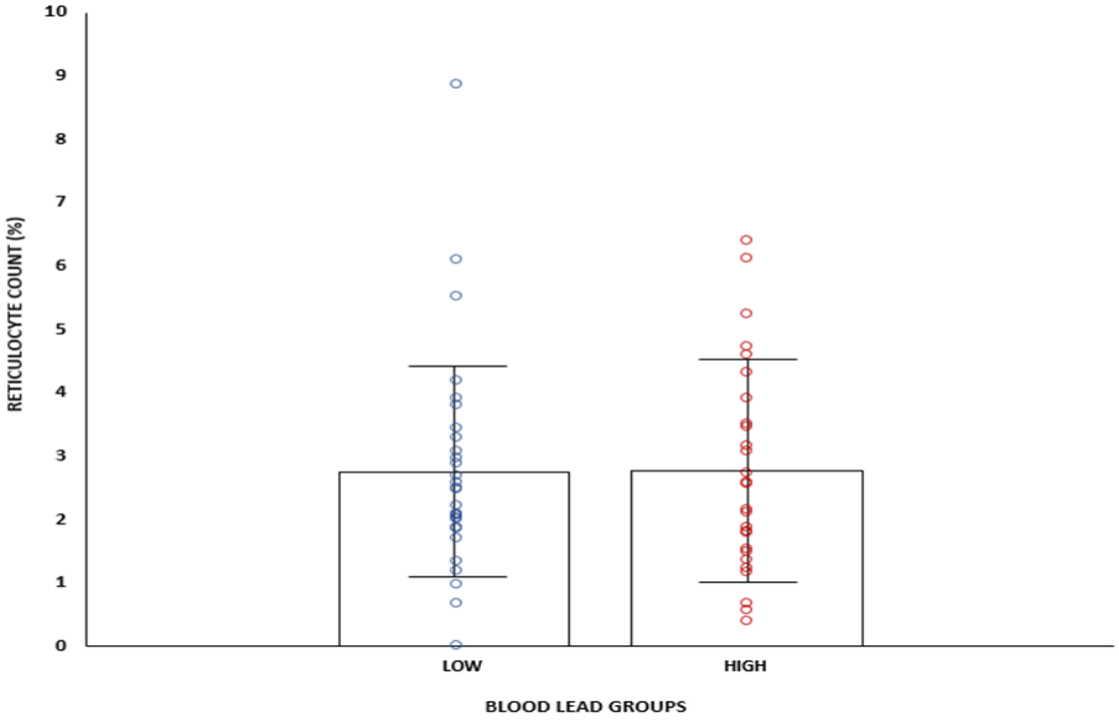

Figure 9. The percentage of reticulocytes in the blood of sparrows with high or low blood lead levels. Samples were split into two groups based on blood lead levels of the individual. The "Low" group (represented by blue markers) is indicative of sparrows with blood lead levels <20µg/dL (n = 29; mean = 2.77±0.33) and the "High" group (represented by red markers) is indicative of sparrows with blood lead levels >90µg/dL (n = 27; mean = 2.76±0.32). Individual data points are presented, along with their mean (height of the bar) ± SD.

Table 5. Linear model final output to examine reticulocyte counts. A significant relationship was seen with sex. Multiple R^2: 0.11. Adjusted R^2: -0.09. F-statistic: 6.49 on 1. Df: 54. P-value: 0.0137.

Reticulocytes						
	Estimate	**SE**	**T value**	**P-Value**	**Confidence Intervals**	
					2.5%	97.5%
Intercept	3.29	0.30	10.97	2.32e-15**	2.69	3.89
Lead	0.003	0.004	0.74	0.47	-0.006	0.01
BCI	-1.01	0.13	-1.15	0.26	-1.89	-0.12
Sex	-1.10	0.43	-2.55	**0.01***	-1.97	-0.23

Trace metals

Across the sample of bloods that were assayed the following metals were recorded as being below detectable limits in the majority of individuals: Chromium, Manganese, Nickel, Arsenic, Cadmium, Tin, Antimony, and Mercury, and therefore they were excluded from the correlational analyses. For the remaining metals where 100% of individuals had detectible limits, except in the case of lead where 78% of individuals had detectible limits, we ran Spearman's correlations between the level of metals recorded in the blood samples of the 41 individuals. All of the correlations between different metals are given in Table 7. Positive, but quite weak correlations were observed between lead and Iron, Phosphorus, Copper and Zinc, while no significant correlation was observed between lead and either Selenium or Rubidium.

Sample 1	***Sample 2***	***N***	***Correlation***	***P-Value***
Iron	Phosphorus	41	0.944	**<0.001**
Copper	Phosphorus	41	0.795	**<0.001**
Zinc	Phosphorus	41	0.638	**<0.001**
Selenium	Phosphorus	41	0.651	**<0.001**
Rubidium	Phosphorus	41	0.443	**<0.001**
Lead	Phosphorus	40	0.504	**<0.001**
Copper	Iron	41	0.661	**<0.001**
Zinc	Iron	41	0.629	**<0.001**
Selenium	Iron	41	0.647	**<0.001**
Rubidium	Iron	41	0.447	**0.003**
Lead	Iron	40	0.536	**<0.001**
Zinc	Copper	41	0.608	**<0.001**
Selenium	Copper	41	0.506	**0.001**
Rubidium	Copper	41	0.392	**0.011**
Lead	Copper	40	0.454	**0.003**
Selenium	Zinc	41	0.533	**<0.001**
Rubidium	Zinc	41	0.496	**0.001**
Lead	Zinc	40	0.554	**<0.001**
Rubidium	Selenium	41	0.370	**0.017**
Lead	Selenium	40	0.242	**0.133**
Lead	Rubidium	40	0.293	**0.067**

Table 7. Spearman correlation matrix output of the relationship between the metals detected across the majority of 41 individuals sampled. The correlations are ordered with respect to their magnitude.

Morphological analyses

We recorded the mass of 209 individuals and the tarsus length of 198 individuals (2021) which gave us residuals (R^2: 0.2168, P-value: <0.001) to use as the BCI for individuals. We recorded fat scores in 200 individuals (88 adult males, 87 adult females, 10 juvenile males and 14 juvenile females) and recorded scores in categories 1-4 only out of the possible 0-5 categories. We saw a significant difference in BCI across the four fat score groups (One-way ANOVA. $F_{3,187}$: 4.02. P-value: 0.008.), with birds in the lowest fat score category having a lower BCI than those in a higher fat score category. We explored the effect of blood lead levels on BCI and saw no relationship (Supplementary T15). No other fixed factors or their two-way interaction were found to be of significance on BCI (all $R^2 < 0.004$, all P >0.366; Table 6). A linear regression was performed using the BCI residuals created and the scale mass index (Peig and Green, 2009). A strong correlation (R^2:0.72) was found between the two, allowing us confidence in our use of BCI residuals for data analysis.

Table 6. Linear model final output to examine BCI. No significant relationship was seen. Multiple R^2: 0.004. Adjusted R^2: -0.0009. F-statistic: 0.82 on 1. Df: 196. P-value: 0.3656.

BCI						
	Estimate	**SE**	**T value**	**P-Value**	**Confidence Intervals**	
					2.5%	97.5%
Intercept	-0.10	0.16	-0.64	0.53	-0.42	0.22
Lead	-0.0004	0.003	-0.13	0.89	-0.007	0.006
Sex	0.21	0.23	0.91	0.37	-0.25	0.66

Discussion

We detected lead in 97% of the house sparrow blood samples that we assayed from Broken Hill (400 individuals), and many of the birds that we caught in the more contaminated parts of the town had blood lead levels (>100 µg/dL) that are considered to be lethal (Franson and Pain, 2011). Given the high extremely elevated blood lead levels that we encountered, we were surprised by a relative lack of clear adverse physiological effects. There were no relationships between blood lead and a body condition index; a measure of immunity – heterophil to lymphocyte ratios; or the reticulocyte count, which is often seen as a measure of stress or oxidative damage to the blood (Stier et al., 2013). The only detectable physiological response to lead that we found with our assays was a negative relationship between blood lead and the concentration of haemoglobin. Whilst this was a linear response across the whole population, it equated to a difference of about 6% in haemoglobin concentration between the sparrows with the highest blood lead levels, and those with negligible levels of lead in their blood. Whilst statistically significant, this difference is relatively small. As no reference range in avian species is available for the lowest acceptable reduction of haemoglobin before the diagnosis of anaemia occurs, it is not clear what the likely further physiological effects of this difference would be.

Whilst little is known about anaemia in avian species, studies in humans suffering from anaemia show reductions in work capacity – defined by aerobic capacity, endurance, and work productivity, presumably due to the reduction of oxygen transport (Haas and Brownlie IV, 2001). Both human and experimental animal studies have previously utilised VO_2 max tests, to show a correlation between haemoglobin levels and/or anaemia, and a reduced aerobic capacity (reviewed in: Haas and Brownlie IV, 2001). This is a widely used assessment of aerobic fitness, measuring the body's maximal capacity to consume oxygen effectively, and is strongly related to circulating haemoglobin levels (Evans, 2002). For example, Perkkio et al. (1985) conducted a study showing a 16% linear decline in VO_2 max in rats that had a 22% reduction in haemoglobin (from 180g/L to 140g/L) in comparison with control rats where haemoglobin was stable. While avian respiratory systems differ to those of mammals, the haemoglobin in both taxa operate comparatively (Ajloo et al., 2002), thus we can assume anaemic effects in mammals and avian species are alike. Further human studies have associated haemoglobin concentration and/or anaemia, with poor cognitive performance and function (reviewed in: Andro et al., 2013). Given the significant reduction in haemoglobin that we have demonstrated in the lead contaminated birds, it seems reasonable to expect that there may be similar adverse consequences in aerobic capacity, physical endurance and cognitive performance, and these would be useful targets for future work in this population.

The effects of increased lead exposure on the level of haemoglobin in the blood, and the abundance of erythrocytes that we have demonstrated here are consistent with the findings of other contaminated avian populations (Belskii et al., 2005, Berglund et al., 2010, Berglund and Nyholm, 2011). However, there are some differences in the magnitude of the response we found, and the approach of these studies. Past studies that have explored the correlation between lead exposure and haemoglobin concentration in birds have mostly focused on nestlings of study species, and used liver sampling for determining lead concentration in individuals, with Berglund and Nyholm (2011) using blood lead levels instead, reporting a negative correlation to occur with haemoglobin concentrations in pied flycatcher (*Ficedula hypoleuca*) nestlings at smelting sites. Belskii et al. (2005) found the average liver lead concentration (21.7 ug/g) in a sample of Pied flycatcher individuals ($n = 22$) to be statistically significant with decreases in haemoglobin levels of 11% ($p < 0.05$) in comparison to sampled nestlings from non-polluted areas. Similarly, Berglund et al. (2010) found mean haemoglobin concentration in Pied flycatcher nestlings (sample size not given) at a lead polluted site to be reduced by 10% in comparison to those nestlings (sample size not given) who had no lead exposure. So, both of these previous studies found slightly larger effects of lead on haemoglobin, with a 10 and 11% reduction in haemoglobin in the most contaminated birds, compared to our reduction of about 6%. So, although we consider our findings broadly consistent with these earlier findings (Belskii et al., 2005, Berglund et al., 2010), it is useful to consider differences in our findings. The main differences between our findings and these studies are that they all observed nestlings, which may perhaps be more sensitive to exposure whereas we sampled adults, with a small number of juveniles. Additionally, Belskii et al. (2005) had a much smaller sample size ($n = 22$) compared to our large sample size ($n = 177$), and Berglund et al. (2010) and Belskii et al. (2005) compared liver lead relationships to haemoglobin levels as opposed to our findings that compared blood lead relationships. This is relevant because typically the levels of lead in the liver are higher than those in the blood (Franson and Pain, 2011) and therefore the contamination levels in these other species are much lower than those in the sparrows that we have studied. The larger sample size of the present study certainly provides greater power than these earlier studies (Belskii et al., 2005, Berglund et al., 2010, Berglund and Nyholm, 2011) based on much smaller sample sizes, in determining the deleterious effect of lead on haemoglobin levels in birds, with likely consequent effects on respiratory function which can result in a decrease in fitness and survival rate (Franson and Pain, 2011).

At a local, and specific level, our finding of the linear relationship between lead and haemoglobin is also useful because it provides an opportunity to assess the potential scale of adaptation to a leaded environment, as suggested by the earlier study of this population (Andrew et

al., 2019). The prevalence of lead in the Broken Hill sparrow population, the relatively weak effect that we found on haemoglobin levels, along with the fact that all of the birds that we sampled were captured using mist nets from a free-living population (and appeared to be in good health and functioning well), supports the idea of some adaptive resistance to lead in this population. It would be useful in further studies to test the adverse effects of lead experimentally on both Broken Hill birds that have and haven't been contaminated, and birds from elsewhere that don't have a recent history of lead contamination. Such tests could be performed by comparing the response in the level of haemoglobin, and that will help us to understand the extent to which individuals in this population may be somewhat adversely affected by lead, and where the population or species fits into the scale of lead poisoning presented by (Franson and Pain, 2011).

Compared to the levels of blood lead reported in other studies in the literature, the levels that we have reported are at the very high end for birds that were sampled whilst apparently healthy (see Table 1). Most higher blood lead levels have been reported in individual birds that were either found dead, or very sick, and that subsequently died (eg. Carneiro et al. 2016). A number of sparrows in the most contaminated area of South Broken Hill had blood levels that are typically thought to be lethal, and whilst we don't know how long these individuals survived after we sampled them, they appeared to have normal levels of health at the time of capture. Whilst relatively high in many individuals, and on average, we are confident that the levels of blood lead we have reported are correct due to the validation of our results with ICP-MS conducted by the Australian National Measurement Institute. This validation allowed us to calculate a correction factor for the values measured by the commercial LeadCare machine, which is known to constantly under-report (Craighead and Bedrosian, 2008, Green et al., 2008, Langner et al., 2015, Gonzalez et al., 2019), but also demonstrated a high relative correlation between the two measures. We also saw low amounts of other trace element contaminants in the sparrow's blood samples, however it is likely that trace amounts of contaminant metals present in samples, as they are essential metals and found alongside lead in the ore body of the local mine. While iron, phosphorus, copper, and zinc were all weakly correlated to lead, we are confident that none contributed any effects on the physiological assays we tested in the sparrows due to the low levels detected.

To further substantiate our lead findings, and specifically the variation in blood lead site averages seen in sites across town (see Figure 1), we can refer back to previous environmental studies of Broken Hill where lead contamination was the focus. Taylor et al. (2014) sampled six playground sites spread throughout the town, to assess the extent of heavy metal contamination exposure on children, and found that the sites closest to the existing mining operations to have the highest levels of lead contamination and upon further isotopic analysis found the lead composition to be

indistinguishable to that of the mining ore body. Our findings in sparrows report the highest individual and site average blood lead values to come from the sampled sites closest to the mining operations (see Figure 1 and 2), demonstrating that our blood levels are reflective of the level of contamination to which individual sparrows are exposed.

The one area in which our sampling was deficient, was that, when tested, some individuals had blood levels that exceed the upper detection range of the LeadCare machine, and therefore, the highest detected blood lead reading was 119µg/dL. There were 19 individual sparrows that, when tested, gave an "HIGH" error message on the LeadCare machine, and presumably all had a higher level than 119µg/dL. As we used the whole 'LeadCare' sample in these failed assays, we were unable to dilute and then try again for these birds. Our future sampling could be slightly improved by diluting samples down by a fixed amount before the assay, or by performing ICP-MS testing for blood lead concentration values on individuals from the most contaminated areas. This work will provide a greater clarity on the blood lead levels of the most contaminated individuals in the population, and increase the average blood lead levels even more, given that the values must exceed the nominal value of 119µg/dL that we assigned to these 19 individuals.

Other than this sampling of the most contaminated birds, we are confident that the sample size of birds sampled overall and across the whole town compare very favourably to other studies of lead contamination in birds. Our sample sizes (2020; n = 218) (2021; n = 182) are significantly larger than many other studies of free-living birds (see sample sizes reported in Table 1). Whilst there are some studies with similar or larger sample sizes, typically they have found lower levels of contamination. For example, in their study of african white-backed vultures *(Gyps africanus),* Garbett et al. (2018) sampled 566 birds exposed to lead in a hunting environment and reported 30.2% of the population to have blood lead levels between 10 and 45µg/dL during hunting season, and 2.3% with blood lead levels >45µg/dL. Another blood lead study performed by Newth et al. (2016) sampled a free-living population of whooper swans exposed to lead during wintering periods, and reported 10% of blood lead levels to be >44µg/dL from 260 individuals. Again, we see that despite a relatively large sample size in a free-living lead exposed population, the number of highly elevated blood lead levels are limited compared to those that we report.

Thus, when we compare these studies to our own, despite all having large sample sizes of free-living populations, we still report greater severity of blood lead within the Broken Hill sparrow population. This is likely due to the long-term high levels of lead exposure in our study allowing a larger proportion of the population to be at risk of elevated lead exposure which translate to increased sub-lethal and lethal blood lead levels, whereas majority of other avian studies sample populations that have been exposed to scarce ingestible lead sources over a short period resulting in

limited individuals with sub-lethal and lethal blood lead values, as seen above in studies by Newth et al. (2016) and Garbett et al. (2018) Based on our findings we can assume that a sparrow living in the South Broken Hill population (ie. the most contaminated part of town, see figure 1 and 2) will almost certainly be contaminated to a high level, that should lead to physiological impairment (Franson and Pain, 2011).

Physiological impairment and external signs of lead poisoning are generally observed when concentrations report in the 50-100 µg/dL range, which is considered clinical or "sub-lethal" lead poisoning (Franson and Pain, 2011). Our findings show that over the whole town (in 2020) 14% (30 / 218) of individuals had blood lead concentrations in this sub-lethal range, with the percentage being higher in the more targeted sample in 2021 (27%; 50/182). In total, we also sampled 34 individuals (4 in 2020 and 30 in 2021) with blood lead levels greater than 100µg/dL, which is considered indicative of severe and lethal poisoning (Franson and Pain, 2011). The increased proportion of these individuals with sub-lethal and lethal blood lead concentrations in 2021 was because we prioritized sampling individuals in known highly contaminated areas based on the 2020 data, to increase our power of detecting the physiological effects of lead contamination. In 2021, when we conducted our physiological assays, our intent was to get a relatively even sample of birds with some of the highest blood lead levels, and those with relatively low blood lead. The distribution of blood leads levels demonstrates that we did achieve this aim, and provided a good sample size of birds in which to test for signs of lead poisoning. This sampling strategy, along with the high concentrations of blood lead in our population again compares favourably with other studies of other avian populations in which adverse outcomes have been reported.

Table 1 summarises the blood lead levels in other avian species in which lead exposure has been studied to provide further context to our findings. For comparison to other studies, we found our average blood lead level for the whole Broken Hill population (sampled in 2020) to be 27.6±1.6µg/dL, with the higher level of 53.2±2.8µg/dL being the 2021 sample that was biased toward the most contaminated areas. As can be seen in Table 1, the average blood lead level reported in µg/dL in the literature range has ranged from 0.6-43.07µg/dL with only 18 out of 58 studies having a higher mean level than 27, and 20 having an average as higher than our 2021 sample, however most of these were from one or a few sampled individuals that had died from ingestion of a lead pellet causing acute exposure which explains such high averages. Whilst our overall 2021 sample had a relatively high average blood lead of around 53 µg/dL, this sample also aimed to contain a balanced number of high and low blood lead levels, and therefore the mean level is reduced by the birds sampled in the areas of low lead contamination. The 68 birds sampled in our two contaminated sites had an average blood lead concentration of 88.1µg/dL. This highlights how

contaminated this sparrow population is, especially in certain areas of town, and indicates why, in comparison to the many other contaminated avian populations, with much lower blood lead levels, that have found physiological effects (reviewed in: Pain et al., 2019) we should have been confident of finding effects if they were present.

Heterophil:lymphocyte ratios are thought to show levels of stress in an individual, as changes in the cell counts are responses to short- and long-term stress in avian species (reviewed in: Minias, 2019). H:L ratios have become a widespread biomonitoring avian studies tool to estimate stress levels in birds due to influences such as heavy metal pollution (Bauerova et al., 2017, Cid et al., 2018, Meissner et al., 2020), disease (Davis et al., 2004), noise (Campo et al., 2005) and urbanization (Ribeiro et al., 2022). Advantages to the technique include its non-destructive nature, ease of sample collection and the extremely small drop of blood needed allows all species to be sampled.

Several studies of birds have demonstrated changes in heterophil and lymphocyte proportions due to environmental heavy metal pollution exposure, even at much lower levels of contamination than in our study (Eeva et al., 2005, Cid et al., 2018) In contrast to these findings, no statistically significant relationship was seen to exist between heterophil/lymphocyte ratio counts and blood lead levels in our samples of the sparrows. In their review, Minias (2019) outlined findings of 415 H:L ratio estimates across many avian species and reported the average sample size 45.7±3.4, which compares favourably with our H:L sample size of 50 individuals. Minias (2019) reports that most of these studies have found a significant response of the H:L ratio to stress, resulting from numerous biotic and abiotic sources. We found no indication that the H:L ratio in the Broken Hill sparrows was linked to blood lead level, and therefore the interpretation is that our study signifies no variation in the stress of high leaded and low leaded in sparrows. This again supports the idea that the Broken Hill sparrows may be resistant in some way to the deleterious, or stressful effects of lead.

In a similar way we found no evidence that high blood lead concentrations caused an increase in the proportion of reticulocytes in this house sparrow population. Furthermore, it has been proposed (Martinho, 2012) that for adult birds a healthy range of reticulocytes would be 1-5%. Despite the very high blood lead levels in our sample, the data from most of the individuals sampled fitted comfortably within that range (Supplementary T1). Again, this is a somewhat unexpected finding, given that previous studies have shown that elevated blood lead reduces the life span of erythrocytes (Franson and Pain, 2011, Haig et al., 2014), and they should therefore be replaced at a higher rate than normal. Additionally, as described above, we did find a slightly reduced level of erythrocytes in the birds with the highest blood lead and while little has been found in regards to the

effects of lead exposure on reticulocyte proportions in avian literature, Belskii et al. (2005) reported heavy metal exposure in pied flycatchers to increase the average percentage of reticulocytes when comparing nestlings from contaminated sites to reference sites. Nestlings from unpolluted reference sites had an average reticulocyte count of 9.1% in comparison to nestlings from the polluted site that had an average of 16.9%, showing a 1.9 factor increase, although statistical analysis was not performed to confirm the correlation between this increase and heavy metals (Belskii et al., 2005). Again, it may be relevant that our study largely focused on adults, and potentially nestlings may either be more susceptible to the effect of lead, or may have a greater capacity to increase the production of erythrocytes. Nevertheless, it was surprising that we found no difference in this useful measure of a response to oxidative damage or systemic stress.

Largely in line with our assays relating to blood physiology (except haemoglobin), we also found no evidence that blood lead levels were affecting overall condition. We used a well-used body condition index in birds, that has become a widely accepted measure of an individual's condition (reviewed in: Labocha and Hayes, 2011). We found that our continuously distributed BCI variable was related to our categorical fat scores, suggesting that both measures were capturing the variation across individuals in an ecologically appropriate trait. In previous studies, individuals with high blood lead levels have been found to have significantly lower body condition (Pain et al., 2019), so again our findings are somewhat surprising. Our findings suggest that the sparrows are able to maintain good levels body fat levels regardless of lead exposure. To establish and maintain good condition, or fat scores, an individual needs to function well in its environment, finding food, foraging efficiently, and optimizing its use of energy. That is why body condition or fat scores are a good indication of evolutionary fitness, and could make individuals with high levels less susceptible to increased predation or starvation, improving survival rates (Scheuhammer and Norris, 1996, Newth et al., 2016). Again, the fact that sparrows with high lead levels are able to apparently function as well as those with low levels again supports the idea that somehow, they are minimizing the usual and widespread deleterious effects of lead.

So, the suite of measures that we have used to address potential lead poisoning in the Broken Hill population has revealed relatively limited evidence for adverse consequences of exposure to levels of lead that would be considered lethal in most other avian populations. Our findings warrant further investigation to understand if there is something special about the species more broadly, or this particular population. House sparrows have been known to cohabit with humans for at least 10,000 years and have been long known as obligate human commensals (Sætre et al., 2012). Over this time, man has evolved industrial processes, ranging from mining to urbanization which involved a plethora of lead use in products and heavy contamination of living environments, all

whilst sparrow populations have continued to establish surviving populations. This suggests the possibility that the house sparrow has become more tolerant to environmental contamination pressures, and perhaps has been adapting in response to lead exposure over that same period of time acquiring some resilience to lead poisoning over thousands of generations

Alternatively, or perhaps alongside a longer-term species-wide response, the evolutionary response may have been very localized and have occurred over a much shorter time frame. The house sparrow population of Broken Hill was established from a relatively small number of founders in 1897 (Andrew and Griffith, 2016) following on from the discover of the ore body and the establishment of the human settlement. The level of environmental lead contamination in the area is still significantly high on a global scale (Taylor et al., 2014) and was even higher in previous years, due to smelting operations occurring in town that have since ceased (Kristensen et al., 2017). The house sparrow population in Broken Hill is effectively genetically isolated from other house sparrow populations, due to the remoteness of Broken Hill, which is isolated by hundreds of kilometers of desert from the nearest towns. This desert is ecologically inhospitable to sparrows, and indeed a recent molecular study has demonstrated that the Broken Hill population is genetically distinct from those in the nearest neighbouring towns in New South Wales and Victoria (Andrew et al., 2017) .The isolated nature of the Broken Hill would mean that any selective pressures acting on the individuals in that population are not being eroded by immigration into the town from outside, improving the efficiency of a locally adapted evolutionary response.

This hypothetical idea of an evolutionary response to the contaminated environment of Broken Hill, is supported by earlier work by Andrew et al. (2019) who used a largescale SNP mapping analysis to identify further showed twelve genes that were suggested to be under selection in Broken Hill and another isolated mining town much further north in Australia (Mt Isa). These 12 genes were argued to be associated with either heavy metal contamination, or metal ion membrane transporters in other taxa (Nebert et al., 2012, Liu et al., 2008, Ding et al., 2014), and could potentially play an important role in supporting the lack of adverse physiological effects of lead, that we report.

Our findings provide support for a much closer examination of lead resilience in this population. It would be useful to identify the lead thresholds that do cause physiological effects in this population, whist also comparing the Broken Hill sparrow population to those elsewhere that live in towns, where exposure to lead has been historically much lower. It would be useful to establish reference data for the species across multiple towns in Australia with respect to haemoglobin concentration and packed cell volume, heterophile/lymphocyte ratios and reticulocyte counts. This would permit the further exploration of potential genetic resistance occurring in the

lead exposed populations of other isolated mining towns (where lead is a significant contaminant). Furthermore, studies of the Broken Hill sparrow population focusing on blood lead levels and their physiological effects in nestlings would also be beneficial as it is possible that more adverse effects could occur in nestlings rather than adults of the population. Additionally, we could experimentally lead-dose individuals taken from different backgrounds, providing the ultimate experiment test of whether an adaptive response can explain our findings. These future studies could also examine the genotypes of individuals with respect to their resilience, and also examine gene expression in individuals experimentally exposed to lead. Such work will provide a nice examination of evolutionary capacity of organisms to adapt to one of the major environmental signatures of the Anthropocene, as well as helping to highlight the damage that we are doing to the environment.

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Supplementary materials

Supplementary T1. Full data set of individuals caught during April-May sampling period 2021.

Band number	Sex	Age	Mass (g)	Tarsus length (cm)	Blood lead (µg/dl)	Transformed blood lead (lc*1.8296)	Haemoglobin (g/l)	H:L Ratio	Reticulocytes (%)	Fat score
3746657	F	A	24.82	18.71	40.8	74.6	162	-	-	2
3746683	F	A	27.69	19.37	48.7	89.1	159	-	-	2
3746692	M	A	25.51	20.05	60.6	110.9	185	-	2.14	2
3746699	M	A	27.79	18.02	24.5	44.8	119	-	-	3
3778641	M	A	23.37	19.15	10.4	19.0	189	-	-	3
3778667	M	A	26.4	18.93	10.9	19.9	184	-	-	2
3778674	F	A	24.73	18.79	12.2	22.3	194	-	-	2
3778693	F	A	28.21	20.23	3.6	6.6	166	-	-	2
3778849	F	A	25.62	19.66	5.8	10.6	165	0.8	2.58	1
3778852	M	A	25.33	20.33	5.5	10.1	175	0.21	3.07	2
3778855	M	A	22.3	18.42	4.5	8.2	199	0.36	1.19	2
3779004	M	A	26.94	18.21	-	-	-	-	-	2
3779031	M	A	26.82	19.58	44.6	81.6	160	-	-	2
3779058	M	A	24.68	17.48	23	42.1	168	-	-	2
3779175	M	A	26	18.95	-	-	-	-	-	-
3779176	M	A	26	20.05	-	-	-	-	-	-
3779177	F	A	25	19.1	-	-	-	-	-	-
3779178	M	A	29	18.5	-	-	-	-	-	-
3779179	M	A	20	19.35	-	-	-	-	-	-
3779180	M	A	23	18.2	-	-	-	-	-	-
3779181	M	A	21	18.1	-	-	-	-	-	-
3779182	F	A	23.5	19.78	-	-	-	-	-	2
3779183	M	A	26.77	19.46	-	-	-	-	-	2
3779184	M	A	29.2	19.75	-	-	-	-	-	3
3779185	F	A	23.77	19.15	-	-	-	-	-	1
3779187	M	A	26.32	20.21	-	-	-	-	-	1
3779188	M	A	27.4	20.6	-	-	-	-	-	1
3779189	M	A	28.59	19.94	-	-	-	-	-	1
3779190	F	A	25.26	19.63	-	-	-	-	-	3
3779191	F	A	27.41	19.78	-	-	-	-	-	2
3779192	F	A	26	19.46	-	-	-	-	-	2
3779193	F	A	28.72	19.56	9.3	17.0	161	3.15	2.06	3
3779194	M	A	24.5	20.01	25.4	46.5	155	-	-	3
3779195	F	A	25.87	19.81	15.1	27.6	184	-	-	2
3779196	M	A	26.19	19.6	23.3	42.6	156	-	-	1
3779197	M	A	26.32	19.34	45.6	83.4	127	-	-	1
3779198	F	A	23.32	18.06	54.3	99.3	148	1.68	4.59	3
3779199	M	A	24.42	18.63	19.8	36.2	152	-	-	2
3779200	M	A	27.15	19.95	-	-	-	-	-	2
3779202	F	A	24.36	18.7	12	22.0	163	-	-	2
3779204	M	A	23.56	18.48	8.2	15.0	179	1.62	-	2
3779228	F	A	22.67	18.87	18.2	33.3	136	-	-	1
3779258	M	A	26.75	19.71	17.4	31.8	160	-	-	3
3779259	M	A	27.9	19.97	20.1	36.8	156	-	-	2
3779260	M	A	25.04	20.67	17.4	31.8	133	-	-	2
3779261	M	A	24.51	20.03	26.58	48.6	145	-	-	1
3779262	M	A	25.17	-	65.1	119.1	127	0.17	-	3
3779263	M	A	25.01		21.3	39.0	171	-	-	2
3779264	M	A	24.58	-	24.8	45.4	178	-	-	2
3779265	-	J	24.92	-	15.3	28.0	155	-	-	1
3779266	M	A	26.05	-	25.1	45.9	162	-	-	2
3779267	F	J	26.29	-	23.1	42.3	155	-	-	2
3779268	M	A	24.49	-	16.1	29.5	186	-	-	3
3779269	M	A	27.84	-	34.2	62.6	184	-	-	3
3779270	M	J	26.82	-	29.7	54.3	141	-	-	2

3779271	F	A	25.05	19.69	10.7	19.6	163	-	-	2
3779272	M	A	26.56	19.73	8.2	15.0	189	-	3.29	3
3779273	F	A	27.62	19.59	17.5	32.0	172	-	-	4
3779274	M	A	25.58	20.42	27	49.4	188	-	-	3
3779275	M	A	25.42	19.53	2.6	4.8	165	-	-	3
3779276	F	A	27.46	20.02	9.8	17.9	181	-	3.91	2
3779277	F	A	28.05	20.52	8.6	15.7	172	-	2.21	1
3779278	M	J	24.59	18.82	5.1	9.3	156	-	-	2
3779279	M	A	26.93	19.8	6.4	11.7	152	-	0.00	1
3779280	M	A	28	21.49	7.5	13.7	144	-	3.80	2
3779281	F	A	23.42	20.36	8.9	16.3	181	-	6.10	2
3779282	F	J	25.94	20.46	7.2	13.2	171	-	-	3
3779283	F	A	22.11	17.9	8.1	14.8	151	3.73	2.88	1
3779284	F	J	27.37	20.4	8.5	15.6	181	2.33	-	3
3779285	M	A	23.13	19.22	12.4	22.7	176	-	-	1
3779286	F	A	25.92	20.35	3.5	6.4	171	-	1.69	1
3779287	F	A	25.26	19.61	4.2	7.7	175	0.5	0.96	1
3779288	F	A	22.29	17.34	16.9	30.9	153	-	-	2
3779289	F	A	25.38	18.63	38.3	70.1	161	-	-	3
3779290	F	A	24.45	19.25	65.1	119.1	149	1.23	6.39	2
3779291	F	A	26.91	19.53	49.4	90.4	170	-	3.45	3
3779292	M	J	26.45	20.63	46.2	84.5	154	-	-	2
3779293	M	A	25.83	21.03	24.7	45.2	162	-	-	2
3779294	F	A	25.5	20.03	26.5	48.5	169	-	-	3
3779295	M	A	26.07	18.53	64.2	117.5	158	0.89	1.49	3
3779296	M	A	23.86	16.8	63.2	115.6	164	-	5.24	2
3779297	M	A	29.8	20.2	25.1	45.9	150	-	-	2
3779298	F	A	26.69	19.66	42.4	77.6	176	-	-	3
3779299	F	A	24	17.14	45.6	83.4	157	-	-	2
3779301	F	A	24.02	20.91	35.5	65.0	148	-	-	3
3779302	M	A	26.7	20.47	32.9	60.2	171	-	-	2
3779303	F	A	28.18	19.48	65.1	119.1	155	-	2.11	2
3779304	M	A	24.72	19.04	35.6	65.1	165	-	-	3
3779305	F	A	26.2	19.93	49.2	90.0	159	-	-	2
3779306	M	A	24.88	20.22	35.8	65.5	179	-	-	1
3779307	M	A	28.42	20.12	21.8	39.9	137	-	-	2
3779308	M	J	27.75	21.03	26.9	49.2	159	-	-	2
3779309	M	J	24.9	19.67	54.3	99.3	180	1.94	-	2
3779310	M	A	27.33	20.89	41.7	76.3	173	-	-	1
3779311	M	A	25.08	19.89	65.1	119.1	159	0.28	2.72	3
3779312	F	A	21.95	18.76	8.8	16.1	165	-	8.86	2
3779313	F	A	25.3	17.86	55.8	102.1	160	1.04	3.91	3
3779314	M	A	21.93	18.34	5.2	9.5	131	0.24	1.86	2
3779315	F	A	21.82	18.31	12.6	23.1	157	-	-	2
3779316	F	A	24.84	19.94	14	25.6	178	-	-	1
3779317	M	A	25.7	20.57	14.9	27.3	181	-	-	2
3779318	F	A	25.4	18.73	3.3	6.0	147	2.68	2.02	3
3779319	F	A	22.95	18.33	15	27.4	195	-	-	2
3779320	F	A	24.6	19.57	10.1	18.5	161	-	5.51	2
3779321	F	A	27.9	20.16	5.6	10.2	169	2.67	2.68	1
3779322	M	J	22.38	19.3	65.1	119.1	130	0.32	-	1
3779323	F	A	26.22	19.27	14.5	26.5	159	-	-	2
3779324	M	A	23.58	19.11	13.7	25.1	191	-	-	1
3779325	M	A	23.35	18.16	11.8	21.6	196	-	-	1
3779326	F	A	23.8	19.13	24.3	44.5	185	-	-	1
3779327	F	A	25.89	19.86	11.8	21.6	161	-	-	1
3779328	F	A	26.44	20.82	18.7	34.2	182	-	-	2
3779329	F	A	25.07	19.69	14	25.6	165	-	-	2
3779330	F	A	24.53	18.39	11.2	20.5	170	-	-	2
3779331	F	A	25.44	19.42	49.2	90.0	150	-	-	2

3779332	F	A	28.81	19.63	11.8	21.6	157	-	-	1
3779333	M	J	26.96	19.57	47.2	86.4	174	-	-	3
3779334	F	J	27.48	20.7	17.7	32.4	160	-	-	3
3779335	F	J	28.31	19.65	13.2	24.2	163	-	-	2
3779336	F	J	28.08	20.14	65.1	119.1	150	-	-	3
3779337	F	J	25.89	18.78	13.4	24.5	134	-	-	2
3779338	F	J	26.25	19.23	11.2	20.5	163	-	-	1
3779339	M	J	25.47	19.08	27	49.4	164	-	-	2
3779340	M	A	26.33	20.68	16.4	30.0	170	-	-	2
3779341	M	A	28.23	19.36	57.3	104.8	159	-	1.22	1
3779342	F	A	24.51	20.32	14.5	26.5	160	-	-	1
3779343	M	A	25.36	18.95	21.6	39.5	185	-	-	2
3779344	M	A	24.92	20.05	27.7	50.7	160	-	-	2
3779345	F	A	24.28	19.92	7.1	13.0	134	0.32	1.33	1
3779346	M	A	25.44	21.05	36.4	66.6	163	-	-	2
3779347	M	A	26.51	19.99	19.4	35.5	187	-	-	1
3779348	F	A	24.92	19.21	23.9	43.7	179	-	-	2
3779349	F	J	26.66	19.19	19.1	34.9	170	-	-	3
3779350	M	A	25.95	20.72	11.1	20.3	172	-	-	2
3779351	F	A	26.12	19.53	5.7	10.4	172	0.5	2.96	2
3779352	M	J	25.42	19	5.1	9.3	181	0.46	-	3
3779353	M	A	26.12	20.27	19.5	35.7	150	-	-	1
3779354	F	J	25.56	18.74	16.8	30.7	168	-	-	2
3779355	M	A	26.74	20.77	41.6	76.1	162	-	-	2
3779356	F	A	26.33	19.7	49.6	90.7	152	1.5	-	2
3779357	M	J	25.02	20.73	56.4	103.2	185	-	-	1
3779358	F	A	23.28	17.48	65.1	119.1	151	0.32	-	2
3779359	M	A	24.21	18.85	65.1	119.1	111	0.04	-	1
3779360	F	A	24.5	17.36	38.7	70.8	167	-	-	2
3779361	M	A	25.16	19.93	62.2	113.8	166	0.45	0.67	2
3779362	F	A	24.98	20.1	56.6	103.6	143	0.39	3.50	1
3779363	M	A	26.02	20.33	65.1	119.1	156	-	3.16	2
3779364	F	A	28.14	20.35	58.4	106.8	148	-	6.12	3
3779365	M	A	28.86	19.03	65.1	119.1	110	2.64	1.52	2
3779366	M	A	27.57	20.13	51.5	94.2	159	-	2.56	1
3779367	M	A	27.29	20.35	50.8	92.9	173	-	1.17	2
3779368	F	A	23.17	19.65	54.5	99.7	182	-	-	1
3779369	F	A	21.45	18.74	65.1	119.1	175	-	-	1
3779370	M	A	24.91	19.58	57.8	105.8	186	-	1.88	2
3779371	F	A	24.31	18.81	57.3	104.8	167	1.48	2.57	1
3779372	F	A	23.9	20.15	33	60.4	183	-	-	2
3779373	F	A	24.23	19.17	51.4	94.0	185	2.14	1.78	2
3779374	M	A	26	18.87	51.1	93.5	171	2.93	0.39	1
3779375	F	A	25.1	20.8	34.1	62.4	164	-	-	2
3779376	M	A	25.28	18.46	65.1	119.1	159	1.9	4.73	2
3779377	M	A	26.44	18.94	65.1	119.1	168	3.77	0.57	3
3779378	M	A	27.25	19.97	35.8	65.5	156	-	-	1
3779379	F	A	25.24	19.54	65.1	119.1	164	1.31	1.81	2
3779380	F	A	29	20.02	41.3	75.6	148	-	-	4
3779381	M	A	25.31	18.73	47	86.0	184	-	-	3
3779382	F	A	24.39	18.5	37.5	68.6	170	-	-	2
3779383	M	A	23.25	19.53	28	51.2	196	-	-	2
3779384	M	A	24.34	18.68	42	76.8	160	-	-	1
3779385	M	A	26.9	18.95	49.3	90.2	162	-	4.32	2
3779386	F	A	23.5	19.59	65.1	119.1	106	-	1.35	3
3779387	F	J	24.64	19.73	37.3	68.2	155	-	-	1
3779388	F	J	24.19	18.83	56.5	103.4	161	-	-	3
3779389	F	A	23.45	19	65.1	119.1	155	0.44	-	2
3779390	F	A	24.9	18.25	46.6	85.3	161	-	-	2
3779391	F	A	23.81	18.41	26.3	48.1	166	-	-	1

3779392	M	A	24.74	19.99	47.3	86.5	161	-	-	2
3779393	F	A	22.71	18.04	41.8	76.5	146	-	-	1
3779394	F	A	25.8	19.13	65.1	119.1	170	0.75	-	2
3779395	M	A	26.53	19.93	18.4	33.7	182	-	-	2
3779396	F	A	24.48	19.34	2.5	4.6	163	0.56	3.43	3
3779397	F	A	27.43	19.43	3.2	5.9	165	1.04	2.46	2
3779398	M	A	25.94	20.02	3.5	6.4	164	3.57	-	1
3779399	F	A	26.26	19.06	4.1	7.5	182	2.05	-	2
3779400	M	A	27.02	20.7	2.2	4.0	147	2.67	0.68	2
3781001	F	J	29.15	20.26	3.1	5.7	161	0.67	-	1
3781002	F	A	25.61	19.63	3.7	6.8	186	3.73	-	3
3781003	F	J	27.16	20.37	2.4	4.4	175	1.61	-	2
3781004	M	A	28.05	19.18	1.7	3.1	173	2.24	2.00	1
3781005	F	A	29.47	19.99	1.7	3.1	166	1.38	2.08	2
3781006	M	A	28.65	19.33	3	5.5	173	1.46	2.50	3
3781007	M	A	29.25	20.78	4.7	8.6	172	3.83	2.07	2
3781008	F	A	27.71	20.25	1.7	3.1	171	0.38	-	3
3781009	M	A	25.72	18.36	34.2	62.6	173	-	-	2
3781010	F	A	24.57	20.35	24.4	44.6	150	-	-	1
3781011	M	A	25.61	18.84	27.7	50.7	158	-	-	2
3781012	M	A	24.71	19.31	10.2	18.7	116	-	1.85	2
3781013	F	A	27.52	-	-	-	-	-	-	-
3781014	F	A	26.55	18.08	43.9	80.3	173	-	-	3
3781015	M	A	29.7	20.31	57.1	104.5	186	1.88	3.08	2
3781016	M	A	25.99	19.89	29.3	53.6	176	-	-	2
3781017	F	A	27.9	19.7	46.4	84.9	181	-	-	3
3781018	M	A	27.82	20.99	-	-	180	-	-	1
3781019	F	A	28.65	20.4	-	-	156	-	-	3
3781020	F	A	24.75	19.4	-	-	158	-	-	2
3781021	F	A	25.59	19.41	-	-	175	-	-	2
3781022	M	A	26.75	19.55	-	-	173	-	-	2
3781023	F	A	25.55	19.89	-	-	193	-	-	3

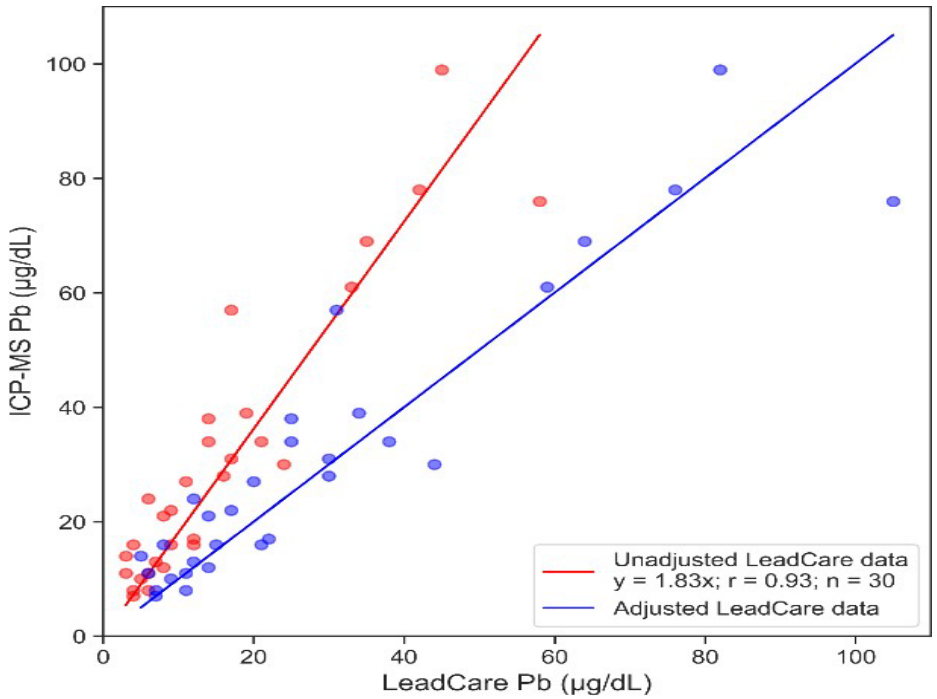
Supplementary T2. Full data set of individuals caught during December sampling period 2021.

2021 - DECEMBER

BAND NUMBER	SEX	AGE	HAEMOGLOBIN (g/L)	PCV (%)
3746579	F	A	167	38
3778832	M	A	169	47
3778859	F	A	163	48
3779113	M	A	135	40
3779363	M	A	156	43
3779399	F	A	180	-
3779400	M	A	183	51
3781026	F	A	173	51
3781027	M	A	159	48
3781030	M	A	161	47
3781037	M	A	142	43
3781039	M	A	175	51
3781063	M	A	149	48
3781065	M	A	164	30
3781067	F	A	166	45
3781068	M	A	179	51
3781070	M	A	170	49
3781072	M	A	179	52
3781073	M	A	192	52
3781074	F	A	140	43
3781075	F	A	171	49
3781076	M	A	156	44
3781077	M	A	185	49
3781078	M	A	172	47
3781079	M	A	187	51
3781080	M	A	184	51
3781082	M	A	178	49
3781083	M	A	183	50
3781086	M	A	174	53
3781088	M	A	179	49
3781089	M	A	175	48
3781090	M	A	187	49
3781091	F	A	163	44
3781094	F	A	166	50
3781096	F	A	129	45
3781099	M	A	169	49
3781101	F	A	182	55
3781102	M	A	173	51
3781104	M	A	180	-
3781106	M	A	158	44
3781107	F	A	173	51
3781109	M	A	175	-
3781110	M	A	151	-
3781111	F	A	152	-
3781113	F	A	149	-
3781114	M	A	173	-
3781115	F	A	160	-
3781131	M	A	156	44
3781132	M	A	158	47
3781135	-	J	134	40
3781136	M	A	166	46
3781138	M	A	186	50
3781139	F	A	132	45
3781141	-	J	102	33
3781143	M	A	169	49
3781146	F	A	164	49
3781147	-	J	143	44
3781148	-	J	157	42
3781149	F	J	162	50
3781150	M	A	156	48
3781151	M	A	145	39
3781152	-	J	142	41
3781153	M	J	177	47

S**upplementary T3.** Full data set of individuals caught during sampling period 2020.

Band number	Sex	Age	Leadcare blood lead (µg/dl)	Converted leadcare (*1.8296)
3746630	F	A	5.7	10.43
3746631	F	A	5.7	10.43
3746632	F	A	6.9	12.62
3746633	M	A	3.1	5.67
3746634	F	A	7.8	14.27
3746635	F	A	7.3	13.36
3746636	F	A	19.5	35.68
3746637	F	A	6.3	11.53
3746639	M	A	5.3	9.70
3746640	F	A	9.8	17.93
3746643	M	A	2.7	4.94
3746644	M	A	12.3	22.50
3746645	M	A	<1.8	3.11
3746646	M	A	49.1	89.83
3746647	F	A	41.8	76.48
3746648	F	A	35.4	64.77
3746649	M	A	32.5	59.46
3746650	M	A	58.1	106.30
3746652	F	A	32.5	59.46
3746656	F	A	44.8	81.97
3746657	F	A	34.2	62.57
3746658	F	A	25.2	46.11
3746659	F	A	36	65.87
3746660	M	A	35.4	64.77
3746663	M	A	25.1	45.92
3778513	F	A	5	9.15
3778514	M	A	7.4	13.54
3778515	M	A	6.2	11.34
3778516	M	A	<1.8	3.11
3778517	M	A	5.7	10.43
3778532	F	A	2.7	4.94
3778533	F	A	<1.8	3.11
3778534	F	A	14.3	26.16
3778535	M	A	24.8	45.37
3778536	M	A	11	20.13
3778537	M	A	21.7	39.70
3778538	M	A	21.1	38.60
3778545	M	A	18.9	34.58
3778546	F	A	11.6	21.22
3778547	F	A	24.8	45.37
3778548	F	A	22.9	41.90
3778549	F	A	36.7	67.15
3778550	M	A	6.3	11.53
3778557	F	A	17.8	32.57
3778558	F	A	13.2	24.15
3778564	F	A	35.8	65.50
3778565	M	A	>65	119.11
3778566	F	A	27.8	50.86
3778568	F	A	38.5	70.44



Supplementary F1. A scatter plot showing the linear data adjustment regression with ICP-MS lead values as the independent variable (y-axis), and their paired LeadCare data as the dependant variable (x-axis). We can see here that this gave us the regression equation y=1.8296 (rounded to y=1.83x for this figure), with r=0.93. This equation was then used to correct LeadCare values in all 2020 and 2021 blood lead data.

Groups	*Count*	*Sum*	*Average*	*Variance*
AD	179	5031	28.11	584.62
JV	39	1022	26.22	258.15

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	114	1	114	0.22	0.64	3.88
Within Groups	113871	216	527			
Total	113986	217				

Supplementary T4. Adults vs Juveniles 2020 blood lead values (adjusted lead used): One-way ANOVA output

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
F	77	2415	31.36	566
M	102	2616	25.66	589

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1430	1	1430	2.47	0.12	3.89
Within Groups	102631	177	579			
Total	104061	178				

Supplementary T5. Males vs Females 2020 blood lead values (adjusted lead used): One-way ANOVA output

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
AD	156	8401	53.86	1429
JV	25	1231	49.25	1418

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	457	1	457	0.32	0.57	3.89
Within Groups	255693	179	1428			
Total	256151	180				

Supplementary T6. Adults vs Juveniles 2021 blood lead values (adjusted lead used): One-way ANOVA output

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
F	91	4576	50.29	1502
M	89	5028	56.50	1346

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	1738	1	1738	1.22	0.27	3.89
Within Groups	253773	178	1425			
Total	255511	179				

Supplementary T7. Males vs Females 2021 blood lead values (adjusted lead used: One-way ANOVA output

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
FAD	77	4037	52.43	1540
MAD	79	4364	55.25	1336

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	309	1	309	0.22	0.64	3.90
Within Groups	221330	154	1437			
Total	221640	155				

Supplementary T8. Males vs Females (juveniles excluded)2021 blood lead values (adjusted lead used): One-way ANOVA output

Supplementary T9. Final output for the analysis of the 2020 catching sites (n=38): One-way Anova. When possible, sites within 500m of each other were combined when <5 blood lead levels were available for Sparrows

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Column 1	2.00	26.60	13.30	4.50
Column 2	5.00	102.70	20.54	14.70
Column 3	5.00	80.80	16.16	70.85
Column 4	15.00	496.00	33.07	232.37
Column 5	6.00	109.30	18.22	57.25
Column 6	5.00	35.30	7.06	24.69
Column 7	6.00	200.70	33.45	55.49
Column 8	6.00	50.90	8.48	31.90
Column 9	5.00	74.30	14.86	23.29
Column 10	5.00	53.60	10.72	2.23
Column 11	5.00	23.70	4.74	13.52
Column 12	6.00	61.80	10.30	7.05
Column 13	5.00	26.20	5.24	4.25
Column 14	5.00	58.50	11.70	3.51
Column 15	5.00	37.30	7.46	0.40
Column 16	5.00	104.70	20.94	164.85
Column 17	6.00	249.40	41.57	106.52
Column 18	5.00	102.30	20.46	141.88
Column 19	5.00	44.90	8.98	8.08
Column 20	5.00	26.50	5.30	6.87
Column 21	5.00	54.90	10.98	72.90
Column 22	5.00	9.90	1.98	0.03
Column 23	6.00	63.30	10.55	13.36
Column 24	15.00	72.70	4.85	6.25
Column 25	5.00	39.00	7.80	28.13
Column 26	5.00	135.00	27.00	148.90
Column 27	5.00	141.40	28.28	123.61
Column 28	5.00	71.70	14.34	36.40
Column 29	5.00	60.80	12.16	15.99
Column 30	6.00	98.80	16.47	575.80
Column 31	5.00	87.90	17.58	15.02
Column 32	6.00	139.00	23.17	208.77
Column 33	5.00	39.00	7.80	13.17
Column 34	5.00	17.90	3.58	3.85
Column 35	8.00	122.50	15.31	56.42
Column 36	5.00	56.90	11.38	30.09
Column 37	5.00	93.10	18.62	88.72
Column 38	5.00	51.30	10.26	37.85

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	20601.1	37.00	556.79	7.48	0.00	1.48
Within Groups	13396.1	180.00	74.42			
Total	33997	217.00				

Supplementary T10. The output for each step of modelling the haemoglobin data with fixed effects and their two-way interaction. Significant relationship was seen with lead and was the only fixed effect in the model for the final output.

Haemoglobin – Full data set

	Estimate	SE	T value	P-Value	Confidence Intervals	
					2.5%	97.5%
Intercept	4858154	183969	26.41	**<2e-16****	4494965	5221343
Lead x BCI	380.8	1548.2	0.25	0.806	-2676	3438
Lead x Sex	2513	5095	0.49	0.62	-7546	12571
Sex x BCI	-173976.4	125274.9	-1.39	0.17	-421303	73350
Sex	147689	193037	0.77	0.45	-233402	528781
BCI	-77393	62129	-1.25	0.21	-200043	-2002
Lead	-7479	2498	-2.99	**0.003****	-12409	-2549

Supplementary T11. The output for each step of modelling the haemoglobin (adult only) data with fixed effects and their two-way interaction. Significant relationship was seen with lead and was the only fixed effect in the model for the final output.

Haemoglobin – Juveniles removed

	Estimate	SE	T value	P-Value	Confidence Intervals	
					2.5%	97.5%
Intercept	4890595	207929	23.52	<2e-16**	4479655	5301535
Lead x BCI	-320.7	1728.9	-0.19	0.85	-3738	3097
Lead x Sex	2969	5603	0.53	0.60	-8106	14044
Sex x BCI	-232391	135565	-1.71	0.09	-500331	35548
Sex	114152	212027	0.54	0.59	-304887	533190
BCI	-78060	67132	-1.16	0.25	-210729	54610
Lead	-8048	2744	-2.93	**0.004***	-13469	-2628

Supplementary T12. The output for each step of modelling the heterophil:lymphocyte ratio data with fixed effects and their two-way interaction. Significant relationship was seen with BCI and was the only fixed effect in the model for the final output.

H/L Ratio – Full data set

	Estimate	SE	T value	P-Value	Confidence Intervals	
					2.5%	97.5%
Intercept	0.03	0.21	0.15	0.88	-0.40	0.46
Lead x Sex	-0.11	0.52	-0.21	0.839	-1.15	0.94
Lead x BCI	0.0004	0.001	0.30	0.76	-0.003	0.003
Sex x BCI	0.28	0.15	1.90	0.06	-0.02	0.58
Lead	0.03	0.24	0.13	0.90	-0.46	0.52
Sex	-0.09	0.24	-0.38	0.71	-0.58	0.40
BCI	0.26	0.07	3.60	**0.0007***	0.12	0.41

Supplementary T13. The output for each step of modelling the heterophil:lymphocyte ratio (adult only) of an individual with fixed effects and their two-way interaction. Significant relationship was seen with BCI and was the only fixed effect in the model for the final output.

H/L Ratio – Juveniles removed						
	Estimate	**SE**	**T value**	**P-Value**	**Confidence Intervals**	
					2.5%	97.5%
Intercept	-0.007	0.22	-0.03	0.97	-0.45	0.44
Lead x Sex	0.31	0.58	0.53	0.60	-0.87	1.49
Lead x BCI	0.0008	0.001	0.48	0.63	-0.003	0.004
Sex x BCI	0.30	0.16	1.85	0.07	-0.03	0.63
Sex	-0.11	0.26	-0.40	0.69	-0.64	0.43
Lead	0.12	0.25	0.46	0.65	-0.40	0.63
BCI	0.28	0.08	3.68	**0.0007****	0.13	0.44

Supplementary T14. The output for each step of modelling the reticulocyte percentage of an individuals with fixed effects and their two-way interaction. Significant relationship was seen with BCI and was the only fixed effect in the model for the final output.

Reticulocytes						
	Estimate	**SE**	**T value**	**P-Value**	**Confidence Intervals**	
					2.5%	97.5%
Intercept	3.29	0.30	10.97	**2.32e-15****	2.69	3.89
Lead x Sex	-0.0005	0.009	-0.05	**0.96**	-0.19	0.02
Lead x BCI	0.002	0.003	0.92	**0.36**	-0.003	0.008
Sex x BCI	0.33	0.26	1.28	**0.21**	-0.19	0.86
Lead	0.003	0.004	0.74	**0.47**	-0.006	0.01
BCI	-1.01	0.13	-1.15	**0.26**	-1.89	-0.12
Sex	-1.10	0.43	-2.55	**0.01***	-1.97	-0.23

Supplementary T15. The output for each step of modelling the BCI of an individual with fixed effects and their two-way interaction. No significant relationship was seen with and fixed effect or two-way interaction.

BCI						
	Estimate	**SE**	**T value**	**P-Value**	**Confidence Intervals**	
					2.5%	97.5%
Intercept	-0.10	0.16	-0.64	0.53	-0.42	0.22
Lead x Sex	0.007	0.006	1.24	0.22	-0.005	0.02
Lead	-0.0004	0.003	-0.13	0.89	-0.007	0.006
Sex	0.21	0.23	0.91	0.37	-0.25	0.66

ANIMAL RESEARCH AUTHORITY (ARA)

AEC Reference No.: 2020/011-4
Online Project ID: 6424

Date of Expiry: 29 March 2022

Full Approval Duration: *30 March 2020 to 27 March 2023*

This ARA remains in force until the Date of Expiry (unless suspended, cancelled or surrendered) **and will only be renewed upon receipt of a satisfactory Progress Report before expiry (see Approval email for submission details).**

Principal Investigator:
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In case of emergency, please contact:
the Principal Investigator / Associate Investigator named above
or the **Animal Welfare Officer** - 9850 7758 / 0439 497 383,

The above-named are authorised by MACQUARIE UNIVERSITY ANIMAL ETHICS COMMITTEE to conduct the following research:

Title of the project: Lead poisoning in the house sparrow in the wild

Purpose: 4 - Research: Human or Animal Biology

Aims: 1. Characterise the genetics of sparrow populations in polluted and unpolluted towns
2. Examine the level of contamination of sparrows with lead and other heavy metals in polluted and unpolluted towns
3. Examine gene expression in a range of tissues as a response to heavy metals

Surgical Procedures category: 3 - Minor Conscious Intervention

All procedures must be performed as per the AEC-approved protocol, unless stated otherwise by the AEC and/or AWO.

Maximum numbers approved (for the Full Approval Duration):

Species	Strain	Age/Sex/Weight	Total		Supplier/Source
17 – Birds Exotic Wild	House Sparrow (*Passer domesticus*)	**Adults/Any/30g**	Banded and Released	1140	Wild
			Euthanased	60	
				1200	

Location of research:

Location	*Full street address*
Broken Hill NSW, Lithgow NSW, Dubbo NSW, Queenstown TAS, Strahan TAS, Zeehan TAS, Roseberry TAS, Wynyard TAS, Mt Isa QLD, Longreach QLD, Noumea New Caledonia, La Foa, New Caledonia	Gardens, in horse stables, and adjacent to hedgerows in which sparrows are aggregating

Amendments approved by the AEC since initial approval:
1. **Amendment – 15/03/2021** – Add Tiarne Harris to protocol (Executive approved. Ratified by AEC 22 April 2021).
2. **Amendment – 15/03/2021 –** Add experimental procedure, technique and/or design (Approved by AEC 22 April 2021).

Conditions of Approval:

1. Limit number of birds caught for Hop-flutter experiments to three per session;
2. At the conclusion of experiments birds must be kept in a dark box for 10 minutes prior to release.

Being animal research carried out in accordance with the Code of Practice for a recognised research purpose and in connection with animals (other than exempt animals) that have been obtained from the holder of an animal suppliers' licence.

A/Prof. Simon McMullan (Chair, Animal Ethics Committee)

Approval Date: 22 April 2021