Title: Unprecedented lead tolerance in an urban lizard

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Abstract

Lead (Pb) is an extremely toxic heavy metal pollutant pervasive in many environments with serious health consequences for humans and wildlife. We found that Cuban brown anole lizards (*Anolis sagrei*) in New Orleans, USA, have the highest mean (955±877 µg/dL; N = 40) and individual (3,192 µg/dL) blood lead levels of any free-living vertebrate reported to date. Unexpectedly, this extreme field lead exposure did not decrease performance in whole-organism traits commonly affected by lead (balance, sprint speed, endurance). To identify toxicity thresholds, we employed a 60-day lead-dosing experiment and found that the brown anole blood lead threshold for decreased whole-organism performance is over an order of magnitude higher than the already extreme mean field concentration (10,600 µg/dL). Transcriptomic analysis of brain and liver from lizards in high and low lead sites revealed minor effects of lead exposure. However, several of the differentially expressed genes function in metal ion homeostasis and oxygen carrying capacity, pointing to cellular mechanisms that may contribute to high lead tolerance in these animals. The brown anole may be the most lead-tolerant vertebrate known to date and has the potential to serve as a powerful model system to help us understand mechanisms of lead tolerance.

Keywords: urban ecology, *Anolis*, heavy metals, locomotion, pollution, ecotoxicology, gene expression

Introduction

Anthropogenic activity has resulted in extensive heavy metal contamination in water, soil, and air, contributing to significant declines in human and wildlife health (Ali and Khan 2019). Lead (Pb) specifically is a major concern due to its potent toxicity (Needleman 2004). Among many effects, lead is neurotoxic and genotoxic, causes neuromuscular disfunction and reduced

cognition, diminished reproduction, anemia, and kidney, liver, and heart disease (Needleman 2004). Reflecting this toxicity, the California condor (*Gymnogyps californianus*) dwindled to near extinction due to lead poisoning in the 1980s (Finkelstein et al. 2012), and the Centers for Disease Control and Prevention (CDC) currently states that no level of blood lead is acceptable for children (CDC 2022). Lead use in paints, gasoline, and industrial processes has been greatly reduced in recent decades (Levin et al. 2021). However, lead does not break down and therefore many cities globally continue to have very high lead contamination due to historical deposition (Clune et al. 2011). As a result, finding organisms that can serve as biomonitors of lead contamination, as well as models of lead susceptibility and tolerance, remains a pressing issue (Tovar-Sánchez et al 2018).

We used the invasive Cuban brown anole lizard (*Anolis sagrei*) in New Orleans, Louisiana, USA, as a model system to investigate effects of urban lead contamination, integrating data on field lead exposure, experimental lead dosing, physiology, and functional genomics. New Orleans provides an ideal location of study because of decades of research into fine-scale geographic variation in soil lead contamination across the city (Figure 1A; Mielke et al. 2016). Soil lead in New Orleans covaries with tissue lead in humans and birds and is associated with detrimental outcomes (Mielke et al. 2016, McClelland et al. 2019, Hitt et al. 2023). Brown anoles are common in cities across the Southeastern United States, including New Orleans, are amenable to both field and laboratory study, and already serve as a model system in urban, physiological, and evolutionary ecology (Battles et al. 2019, Stroud et al. 2019, Deery et et al. 2021). Lizards are underrepresented in studies of heavy metal pollution, especially with respect to lead (Grillitsch and Schiesari 2016). Tissue-environment lead associations are known in some species (Burger et al. 2004, Nasri et al. 2016), but physiological studies of lead exposure in lizards are rare (Holem et al. 2006, Salice et al. 2009, Grillitsch and Schiesari 2016, Indest et al. 2018). Therefore, tissue lead toxicity thresholds in lizards, and by extension their toxicity thresholds relative to better-studied taxa such as birds and mammals, is poorly known.

To test for an association between environmental lead contamination and lizard lead exposure, we measured blood lead concentrations of brown anoles from sites that vary in soil lead level across New Orleans. To determine if field lead exposure affects lizard whole-organism physiology, we tested for associations between blood lead level and multiple metrics of

59 locomotor performance in field-caught animals. To experimentally determine blood lead toxicity
60 thresholds, we measured multiple aspects of brown anole physiology during a 60-day lead dosing
61 study in the laboratory. Finally, to investigate the effects of lead, and potential mechanisms of
62 lead tolerance, at the molecular level, we sequenced transcriptomes of brown anoles from high
63 and low lead contamination areas. We found that brown anoles in New Orleans have extreme
64 blood lead levels and lead tolerance, while gene expression patterns highlight molecular
65 mechanisms that may contribute to tolerance. Brown anoles may therefore be the most lead
66 tolerant vertebrate known to date and provide a compelling vertebrate model system to
67 investigate mechanisms of heavy metal tolerance.

68

69 **Methods**

70 **Field Sampling and Animal Husbandry.** Brown anoles were captured by hand in New Orleans,
71 Louisiana and transported to Tulane University. Animal were housed individually in plastic
72 cages (23 L x 15 W x 16 H cm) with a wooden dowel provided as a perch in fluctuating
73 temperature (35/24ºC, day/night) and humidity (60% daytime, 75% nighttime) controlled
74 Percival chambers on a 14:10 light:dark cycle, conditions that mimic an average summer day in
75 New Orleans (Deery et al. 2021). Lizards were watered twice daily and fed crickets dusted with
76 calcium powder three times per week.

77 **Tissue Lead Analysis.** We measured lead levels in the blood and bone of field animals. Blood
78 samples (~75 µl) were collected from the post-orbital sinus using heparinized capillary tubes
79 (Kimble Chase; Sharma et al. 2014). Each sample was immediately transferred to a trace-metal-
80 clean microcentrifuge tube and stored at -20ºC until processing. For bone collection, animals
81 were euthanized via cervical dislocation and whole femur, tibia, and fibula were dissected out
82 and stored in a trace metal clean micro-centrifuge tubes at -20ºC until processing.

83 Tissue lead levels of lizards collected from 2019 – 2021 were measured at the UC Santa
84 Cruz Plasma Analytical Laboratory following established trace metal clean techniques and using
85 ultra-pure reagents as outlined in Hitt et al. (2023). Blood samples were weighed and then dried
86 overnight at 60ºC to obtain dry weight, then cold digested for 10 hours in 100 µL (blood volume
87 <50 µL) or 150 µL (blood volume >50 µL) of concentrated HNO_3 (optima, Fisher Scientific).

88 30% H_2O_2 (ultrex, JT Baker) and ultrapure water was added to each sample for a HNO_3 to H_2O_2
89 ratio of 2:1 and a final HNO_3 concentration of 6%. Samples were vortexed and left to sit
90 overnight before analysis. Lead concentrations were determined by inductively coupled plasma
91 mass spectrometry (ICP-MS), measuring masses of ^{208}Pb and ^{205}Tl isotopes (used as an internal
92 standard). For quality control, approximately 20 µL of NIST SRM 955c (lead in blood, level 2)
93 was digested using the methods described above with an average recovery of 94% ± 3.4% RSD
94 (N = 4). Frozen bone samples were thawed overnight then combined for each individual and
95 weighed. They were digested for 6 h with sub-boiling concentrated HNO_3 in closed Teflon vials
96 then evaporated to dryness and diluted with 5% HNO_3. Lead concentration was then analyzed as
97 described above.

98 Blood lead levels in lizards collected in 2022 and 2023 were analyzed at the Louisiana
99 Animal Disease Diagnostic Lab. 15 µL of blood were diluted in a solution of 0.1% Triton-X100
100 acidified to 0.2% with HNO_3, resulting in a 21-fold dilution. Seronorm-Trace Elements (lead in
101 blood, level 2, 303 ng/ml Pb) was used as a standard reference material and digested using the
102 methods above with an average recovery of 103% RSD. Clotted samples were first weighed and
103 digested with 600 µL of 35% Trace Metal HNO_3 overnight at 85ºC. Samples were then diluted to
104 a 1/60 solution with 0.2% HNO_3. Lead concentrations were determined using a Perkin Elmer
105 PinAAcle 900Z Zeeman graphite furnace spectrometer.

106 **Whole Organism Performance.** With field-collected animals, we tested for associations
107 between individual blood lead level and performance in whole-organism traits known to be
108 affected by lead exposure in other vertebrates: balance, sprint speed, and endurance (Yu et al.
109 1996, Burger et al. 2004, Mansouri and Cauli 2009, Mansouri et al. 2013). To measure balance,
110 lizards were run on a 1.2 m long horizontal wooden dowel (Spezzano and Jayne 2004) covered
111 with fine mesh. We counted the number of times an individual slipped, defined as anytime the
112 hind legs lost traction such that the pelvis moved to the side of the dowel (Spezzano and Jayne
113 2004). Runs were filmed using an Apple iPhone 8 Plus. Lizards were run twice with 15 min
114 between runs. The dowel was 1.2 m off the ground and foam was placed underneath in case
115 individuals fell or jumped. The diameter of the dowel was 0.79 cm or 0.64 cm for lizards with a
116 knee-to-knee width of ≥ 27 mm and ≤ 26 mm, respectively, to account for inter-knee distance

117 (Spezzano and Jayne 2004). Trials were conducted at 32ºC, the body temperature of maximum
118 field activity for *A. sagrei* in New Orleans (Ryan and Gunderson 2021).

119 Sprint speed was measured by video analysis of lizards running up a 1.8 m wooden track
120 set at a 37º angle and covered with fine mesh for traction (Gunderson et al. 2018). Perpendicular
121 lines were drawn every 12.5 cm and the fastest interval was taken as an individual's maximum
122 speed for each run. The average of the two fastest intervals was used in analyses. Each lizard was
123 run twice at 32ºC and filmed at high speed (120 frames per second) with an Apple iPhone 8 Plus.
124 Runs were 15 min apart.

125 We measured endurance as time to exhaustion (s) while lizards ran at 32ºC on a
126 motorized treadmill (DogPacer MiniPacer) at 0.80 km^{-1} (Lailvaux et al. 2018). Lizards were
127 placed on the treadmill while tethered with thread around the waist and induced to run by gently
128 tapping their tails. Exhaustion was indicated by a loss of righting response. Endurance was
129 measured twice per lizard with 24h between trials and the mean endurance time was used in
130 further analysis.

131 **Lead Dosing Study.** In 2023, N = 50 males were captured at low lead contamination Site B
132 (Figure 1). After one week of lab acclimation, individuals were randomly assigned to one of five
133 treatment groups (N = 10/treatment). Treatments were administered daily by oral gavage (Holem
134 et al. 2006, Salice et al. 2009) for 60 days. The control group received distilled water, while the
135 treatment groups received 1, 10, 100, or 500 mg lead/kg bodyweight, respectively. Following
136 methods previously used in lizards (Holem et al. 2006, Salice et al. 2009, Indest et al. 2018), lead
137 solutions were prepared by diluting lead acetate trihydrate (ThermoFisher Scientific Chemicals)
138 in distilled water (ThermoFisher Scientific Chemicals). We measured seven traits/endpoints:
139 symptomatic lead poisoning, endurance, sprint speed, balance, kidney size, liver size, and testis
140 size (Yu et al. 1996, Burger et al. 2004, El-Said et al. 2008, Mansouri and Cauli 2009, Salice et
141 al. 2009, Gonick 2011, Mansouri et al. 2013, Hora and Wuestefeld 2023). Symptomatic lead
142 poisoning was defined as loss of appetite for one week and/or presence of oral ulcers (El-Said et
143 al. 2008). Animals were removed from dosing if they showed symptomatic lead poisoning.
144 Endurance was measured three times per lizard using the methods described above: the day prior
145 to dosing, after 30 days of dosing, and after 60 days dosing (i.e., the end of the experiment).
146 Individuals removed early due to lead poisoning had their endurance measured on the day of

147 removal. Sprint speed and balance were measured as described above at 60 days on individuals
148 in the control, 1 mg/kg, and 10 mg/kg treatment groups. Blood samples were collected from all
149 individuals prior to the beginning of dosing and after all performance measurements at the end of
150 dosing. Blood lead level did not differ among groups prior to dosing (F-value = 1.05, P = 0.394).
151 Animals were anaesthetized with isoflurane and euthanized via cervical dislocation, after which
152 the liver, kidney, and testes were removed and wet weight was measured with a digital balance.

153 With our dosing data, we calculated the blood lead toxicity threshold for a trait as the
154 highest blood lead level of an individual within the No Observed Effect Concentration (NOEC)
155 treatment for that trait. NOEC is defined as the highest lead dose that does not cause a significant
156 change from the control group (Buekers et al. 2009).

157 **Statistical Analyses of Lead and Performance Data.** All statistical analyses were performed
158 using R Statistical Software (v4.1.2; R Core Team 2021). For the field collected animals, we
159 tested for differences in blood and bone lead level between lizards from high and low lead sites
160 using multiple linear regression with site soil lead level ('high' or 'low') as a predictor variable,
161 with sex and site by sex interaction covariates for blood analyses and sex and snout-vent-length
162 (SVL) as covariates for bone analyses. We tested for an association between blood and bone lead
163 level within individuals with a Pearson product-moment correlation test. We tested for
164 associations between individual blood lead level and endurance and sprint speed with multiple
165 linear regression with sex and SVL as covariates. For tests with balance, negative binomial
166 models were used with sex and relative leg length (residual knee-to-knee width relative to SVL)
167 as covariates.

168 For the dosing study, we first tested for differences in blood lead level among treatment
169 groups prior to dosing with a one-way ANOVA (there were none; P = 0.394). We tested for
170 differences in blood lead level within and among treatment groups due to dosing using a two-
171 way repeated measures ANOVA. One individual in the 500 mg/kg treatment group was found
172 deceased on day 10 of dosing and was excluded from the analyses since a second blood sample
173 could not be collected. We tested for effects of lead dosing on physiological performance at the
174 treatment and individual level. For treatment-level analyses, treatment group was a predictor
175 variable, and for individual-level analyses, individual blood lead level was a predictor variable.
176 For endurance, linear mixed effects models were used with endurance as the response variable,

177 day of measurement and SVL as covariates and individual as a random effect. Two individual-
178 level analyses were conducted for endurance, one with data from all treatments including
179 animals that were removed early due to lead poisoning, and another with only the animals that
180 made it through the entire study. We also tested for differences in sprint speed and balance at the
181 end of the study among the Control, 1 mg/kg, and 10 mg/kg animals at the treatment and
182 individual levels. We tested for differences in balance among treatment groups using a general
183 linear model with a Poisson distribution. We tested for differences in liver, kidney, and testis
184 mass among treatments using one-way ANOVAS. Organ masses were normalized to body mass
185 (organ wet mass/total body mass) before analysis.

186 **Transcriptome Analysis.** To investigate the cellular processes affected by lead exposure, and to
187 provide insight into how the lizards may tolerate high lead levels (see Results), we tested for
188 differential gene expression of lizards collected from a high (Site F) and a low (Site B) lead
189 contamination area in 2021 (Figure 1; N = 5 males and N = 5 females per site). Forebrain (males)
190 and liver (males and females) transcriptomes were sequenced. We chose brain due to the
191 neurotoxicity of lead and liver due to its role in detoxification (Needleman 2004, Hora and
192 Weustefeld 2023). Forebrain was only collected from males due to small female size. After one
193 week in the laboratory under our standard housing regime (see above), individuals were
194 anaesthetized as described above and the target tissues were immediately dissected out, placed in
195 RNAlater, flash frozen in liquid nitrogen, and stored at -80°C. Total RNA was extracted using
196 the Qiagen RNeasy Plus Universal Tissue Mini Kit. Transcriptome libraries were prepared and
197 sequenced at Novogene Corporation Inc (Sacramento, CA) using 150-base pair paired-end
198 sequencing on the Illumina NovaSeq 6000 platform. Sequencing resulted in an average of
199 25,569,255 ± 3,909,485 reads per transcriptome.

200 We used Rcorrector to correct random sequencing errors in reads and removed kmers
201 with errors that were unfixable (Song and Florea 2015). Trimmomatic was used to filter the
202 resultant sequences based on quality, which was assessed for each read with a 4bp sliding
203 window (Bolger et al. 2014). Sequences were trimmed when Phred33 quality scores fell below
204 an average of 15 within the window. Adaptor sequences, if detected, were removed. Quality-
205 controlled sequence reads were mapped to the *Anolis sagrei* genome (AnoSag2.0; Geneva et al.
206 2021) using STAR (Dobin et al. 2013).

We used the DESeq2 package (Love et al. 2014) to identify differentially expressed genes (DEG) between high and low lead groups based on sex and tissue type. We filtered out low expression genes, and only conducted analyses on those with a count threshold of 10 in at least three samples (Love et al. 2014). False discovery rate (FDR) correction for multiple comparisons was applied to maintain a table-wide alpha of 0.05 (Storey and Tibshirani 2003). Genes were considered differentially expressed when the absolute value of log2 fold expression difference between high and low lead groups was ≥ 1 and FDR was <= 0.05 (Love et al. 2014). Volcano plots were generated using EnhancedVolcano (version 1.16.0; Blighe et al. 2022). Gene Ontology enrichment analyses were conducted on differentially expressed genes using Enrichr (Kuleshov et al. 2016).

Results and Discussion

Field Lead Exposure. Lizard blood lead levels varied with soil lead contamination across New Orleans (Figures 1A, B). In the two sites with the most data (low lead contamination Site B and high lead contamination Site F; N = 40/site), blood lead in the high contamination site ($\bar{x}$ ± SD; 955±877 µg/dL) was over 23 times higher than that of lizards from the low contamination site (40.4 ± 50.6 µg/dL; P = < 0.001; Figure 1B; Table S1). Additionally, blood lead levels across the city frequently exceeded the reported toxicity thresholds (Buekers et al. 2009) for whole-organism physiological effects in birds (71 µg/dL) and mammals (18 µg/dL; Figure 1B). The highest individual blood lead level was 3192 µg/dL, which is 2.3 X higher than the highest individual blood lead levels that we could find reported for an individual free-ranging vertebrate, a griffon vulture (*Gyps fulvus*) that died within 24 hours of capture (Carneiro et al. 2016; Figure 1B).

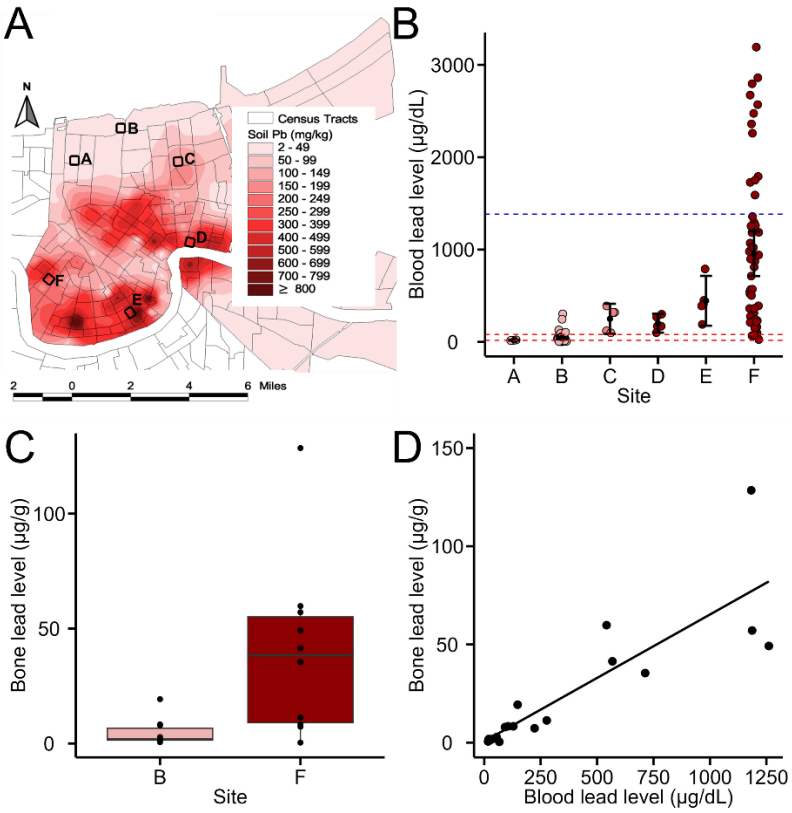

Figure 1. Soil and lizard lead levels in New Orleans. (A) Soil lead concentrations in New Orleans[8] (Mielke et al. 2016) with our study sites indicated. (B) Blood lead concentrations of brown anoles by study site. Dashed red lines: reported whole-organism physiological toxicity thresholds for birds (71 µg/dL) and mammals (18 µg/dL; Buekers et al. 2009). Blue dashed line: the highest individual blood lead level from a live free ranging vertebrate that we could find in the literature (1384 µg/dL; griffon vulture (*Gyps fulvus;* Carneiro et al. 2016). (C) Bone lead levels of lizards collected from Sites B and F. (D) Relationship between bone and blood lead levels for the subset of individuals from Sites B and F for which we have both measures.

Blood lead reflects shorter-term exposure because lead is absorbed into the bloodstream within hours of ingestion and has a half-life that ranges between 3 and 30 days in wildlife and humans (Elder et al. 2014). To determine if the lizards are exposed to lead over longer timescales we measured lead in bone, which begins incorporation during development and can remain for years without further exposure or continue to increase as exposure continues and further

incorporation occurs (Needleman 2004). Bone lead was significantly higher in high contamination Site F (39.9 ± 38.1 μg/g) than in low contamination Site B (4.67 ± 5.83 μg/g; P = 0.012; Figure 1C), indicative of long-term exposure in high contamination areas. Furthermore, individual bone and blood lead levels were significantly correlated ($r = 0.86$, $t_{(18)} = 7.16$, $P < 0.001$; Figure 1D), consistent with lizards residing within their respective sites throughout their lives. Indeed, wild brown anoles rarely move more than a few meters from their natal site (Calsbeek 2009) and adults have small home ranges (mean = 17.6 m^2 for males; Schoener and Schoener 1982). Such high philopatry and adult site fidelity is the norm for *Anolis* lizards generally (Schoener and Schoener 1982), and therefore these lizards (and perhaps lizards more broadly; Talent et al. 2002) are strong candidates to serve as biomonitors of environmental lead contamination.

To place the blood lead levels of brown anoles in New Orleans into a broader context, we compiled all mean blood lead levels we could find reported for live, free-living adult vertebrates (minimum sample size N = 10). If multiple values were found for the same species, only the highest mean value was included. Brown anoles in New Orleans are clearly exceptional (Figure 2). The mean lead level in Site F is over three times higher than the next highest mean value (Nile crocodiles, $\bar{x} = 250$ μg/dL; Humphries et al. 2022), and 10 times higher than the peak mean level in California condors that experienced population declines due to lead exposure (130 μg/dL; Finkelstein et al. 2012; Figure 2). How these lizards attain lead is currently unknown. High lead levels in crocodiles and scavenging birds like condors and vultures are linked to the ingestion of large pieces of lead associated with fishing and hunting equipment (Pain and Mateo 2019, Humphries et al. 2022). That pathway of lead exposure is not possible for brown anoles as small (adult mass = 1.5 – 8 g) insectivores that feed on live prey. Instead, they must ingest it from a combination of diet, drinking water, and breathing or ingesting lead-contaminated dust. Arthropods do accumulate lead in lead-contaminated areas (Khan 2019), although a significant portion of that lead may be stored in the exoskeleton in a form not bioavailable to predators (Inouye et al. 2007). Ingestion of lead in dust is a very common means of lead exposure for humans and wildlife (Mielke et al. 2016), and brown anoles spend the majority of their time near and on the ground in close proximity to contaminated soils.

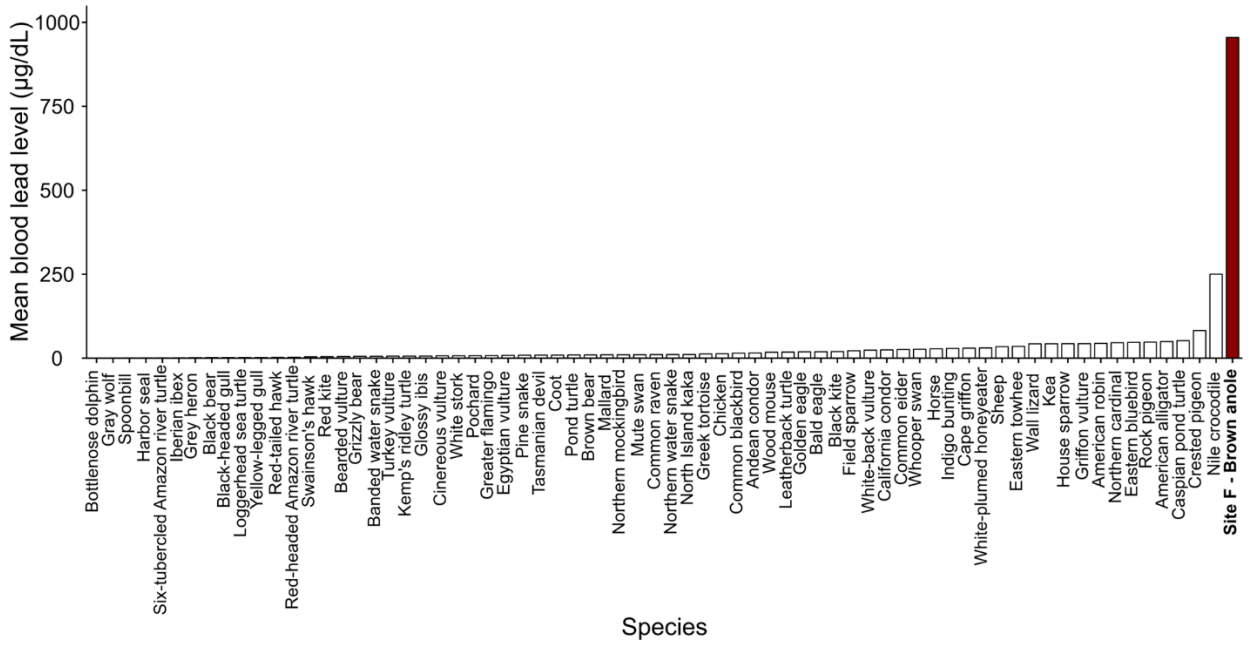

Figure 2. Mean blood lead concentrations of free-living vertebrates. The brown anole value is from high lead contamination Site F (Figure 1). Data for other taxa were reported in the literature (minimum N = 10). If multiple values for a species were found we included only the highest. See Dataset S1 and References S1 for more information.

Physiological Performance of Field Exposed Lizards. Given their high lead levels, we predicted that field-exposed lizards would experience physiological detriments. Contrary to our expectation, blood lead was not associated with decreased performance (Figure 3). Blood lead was unrelated to balance ($P = 0.276$; Figure 3A; Table S2), positively related to sprint speed ($P = 0.01$; Figure 3B; Table S3), and trended towards a positive relationship with endurance ($\beta = 0.04$; $P = 0.086$ Figure 3C; Table S4). Brown anoles in New Orleans therefore appear to have greater lead tolerance than birds and mammals (Buekers et al. 2009). However, these data do not allow us to estimate their blood lead thresholds because no performance detriments were observed. To estimate brown anole lead toxicity thresholds, we turn to our experimental lead dosing experiment.

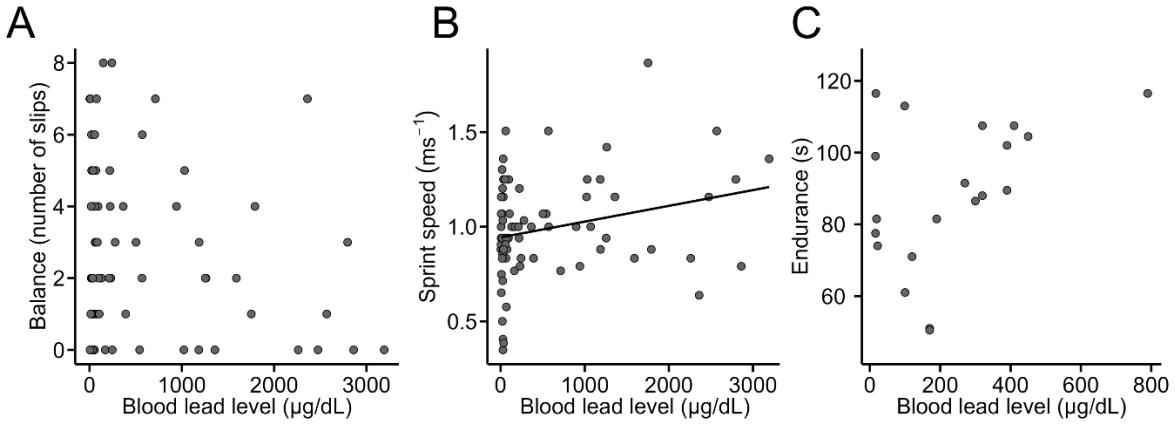

Figure 3. Physiological performance of individual field-caught brown anoles relative to blood lead level. (A) Balance. (B) Sprint speed. (C) Endurance.

Effects of Experimental Lead Dosing. Blood lead level increased in all groups over time consistent with treatment level (Figure 4A, Tables S5, S6). The final mean blood lead level of lizards in all of the lead-dosed treatments was higher than the mean blood level at our most contaminated field site (Figure 4A, Table S5). All lizards in the 100 and 500 mg/kg treatments exhibited symptomatic lead poisoning before the end of the experiment and were removed from dosing by day 40 and day 10, respectively. In contrast, no lizards exhibited symptomatic lead poisoning in the Control, 1 mg/kg, or 10 mg/kg treatments. The NOEC for symptomatic lead poisoning is therefore 10 mg/kg, and the blood lead toxicity threshold is 10,600 µg/dL (Table 1).

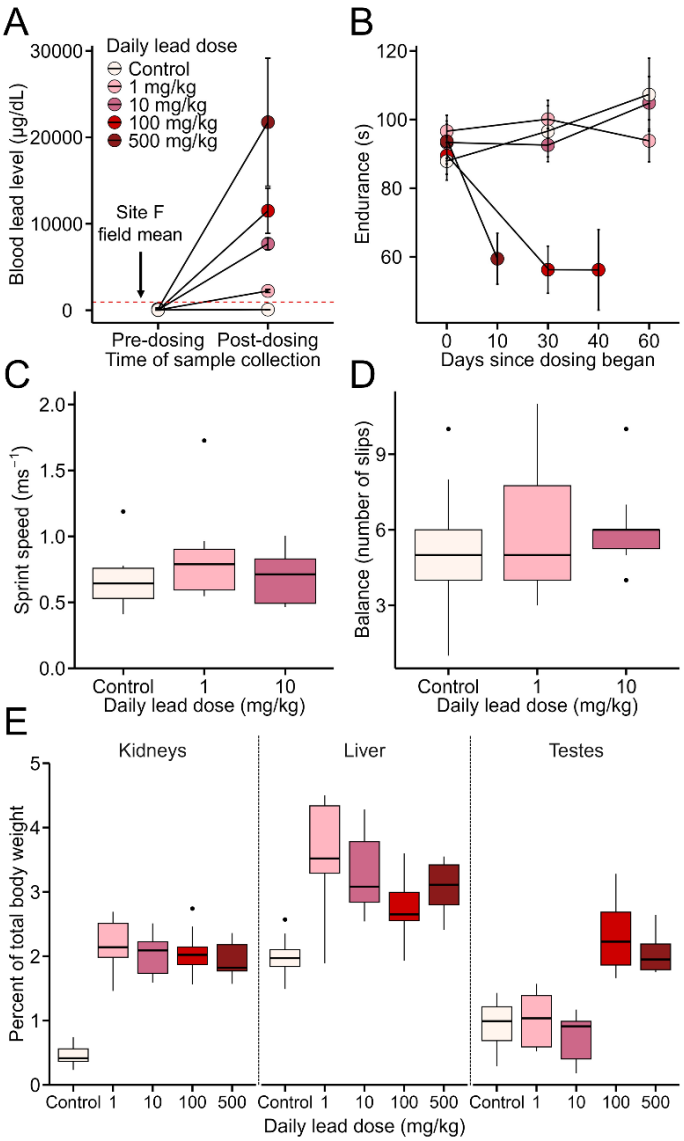

Figure 4. Effects of lead dosing on brown anole lizards. (A) Blood lead concentrations before and at the conclusion of dosing. Post-dosing samples were collected at 60 days except for the individuals removed early due to symptomatic lead poisoning in the 100 mg/kg and 500 mg/kg treatment groups. Points and bars indicate means and standard errors, respectively. The dashed line indicates the mean blood lead level of field-caught brown anoles from Site F, which had the highest lead level of all field sites. (B) Locomotor endurance by treatment group over time. (C) Sprint speed and (D) balance of lizards in the Control, 1 mg/kg, and 10 mg/kg treatment groups at the conclusion of dosing. (E) Organ wet weight as percent of total body mass by treatment.

321 **Table 1. Brown anole lead toxicity thresholds from the dosing experiment.** NOEC = No
322 Observed Effect Concentration. Blood lead toxicity threshold = highest individual blood lead
323 concentration in the NOEC treatment. We do not have a direct estimate of NOEC for kidney and
324 liver size because both organs were enlarged in the lowest-dose treatment, and we report the
325 highest individual blood lead concentration in the Control treatment as the toxicity thresholds for
326 both.

Endpoint	NOEC (mg/kg)	Blood lead toxicity threshold (µg/dL)
Symptomatic lead poisoning	10	10,600
Endurance	10	10,600
Sprint speed	10	10,600
Balance	10	10,600
Kidney size	< 1	130
Liver size	< 1	130
Testis size	10	10,600

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328

329 Endurance differed by treatment ($F = 4.81$, $P < 0.001$), decreasing over time in the 500
330 mg/kg and 100 mg/kg treatments, but not in the others (Figure 4B). The NOEC for endurance is
331 10 mg/kg, and the estimated blood lead toxicity threshold is 10,600 µg/dL (Table 1). We also
332 tested for an association between final individual blood lead level and final endurance. With data
333 from all treatments, blood lead is negatively associated with endurance (blood lead range = 53 –
334 80,000 µg/dL; $R^2 = 0.47$, $\beta = -0.007$, $P < 0.001$; Figure S1A, Table S7). However, when animals
335 from the treatments above NOEC were excluded, endurance and blood lead were unrelated
336 (blood lead range = 53 – 10,600 µg/dL; $R^2 = 0.05$, $\beta = 0.001$, $P = 0.432$; Figure S1B).

337 Sprint speed and balance were unrelated to lead for the only animals that remained at the
338 end of the experiment (the Control, 1 mg/kg, and 10 mg/kg groups) at both the treatment (both P
339 > 0.325; Figures 4C, D; Tables S8, S9) and individual blood lead levels (both P > 0.781; Figures
340 S1C, D; Tables S8, S9). We therefore cannot directly calculate NOEC or blood lead toxicity
341 thresholds for these traits, though we can say that the NOEC and blood lead threshold are at least
342 as high as for endurance (Table 1).

343 Given the dosing results, it is unsurprising that we found no whole-organism
344 physiological detriments of what at first appeared to be very high levels of field lead exposure
345 (Figure 3). The whole organism performance of these animals is not affected until blood lead
346 concentration is much higher than field levels. In our site with the highest lead levels, Site F, the
347 mean blood lead level was 955 µg/dL and the highest documented individual blood lead level
348 was 3192 µg/dL (Figure 1). However, we only began to see whole organism performance effects
349 for individuals in the 100 mg/kg treatment, which had a mean blood lead level 12x higher than
350 the field mean (Figure 2A, Table S5).

351 All lead-dosed treatments had larger kidneys (F-value = 56.47, $P < 0.001$) and livers (F-
352 value = 12.79, $P < 0.001$) than Controls, including the 1 mg/kg and 10 mg/kg groups that
353 exhibited no whole-organism evidence of lead effects (Figure 4E). In contrast, testes were only
354 enlarged in the 100 mg/kg and 500 mg/kg treatments (F-value = 29.50, $P < 0.001$; Figure 4E).
355 The NOEC for effects on liver and kidney size are therefore lower than for whole-organism traits
356 and cannot be directly estimated, but we know the NOEC must be less the 1 mg/kg (Table 1).
357 We report the blood lead toxicity threshold as 130 µg/dL, the highest individual concentration in
358 the Control group (Table 1). Whether or not the liver and kidney responses reflect an adaptive
359 response to lead or pathology are unclear and require further investigation. The kidney and liver
360 remove and sequester heavy metals (Gonick 2011, Hora and Wuestefeld 2023) and increased
361 organ mass may improve detoxification (Hora and Wuestefeld 2023). However, enlarged organs
362 can also reflect heavy metal-induced tissue damage (Habeebu et al. 2000, Gonick 2011). The
363 response of testis mass to dosing was similar to that of whole-organism traits, and had the same
364 thresholds (NOEC = 10 mg/kg; blood lead = 10,600 µg/dL; Table 1). Testes do not generally
365 function in heavy metal detoxification or adaptive sequestration, so changes in testis mass likely
366 reflects harm from lead exposure (Salice et al. 2009, Heidaeri et al. 2021).

367 **Differential Gene Expression of Field Exposed Lizards.** The high lead tolerance of brown
368 anoles raises the questions of how this tolerance is achieved. Lead exposure in vertebrates
369 typically causes the differential expression of hundreds if not thousands of genes within a given
370 tissue (Schneider et al. 2012, Shi et al. 2020). Genes commonly affected by heavy metal
371 exposure are those related to the cellular stress response (e.g., antioxidants and molecular
372 chaperones; Needleman 2004, Gupta et al. 2010), transport of key ions that heavy metals can

replace within biological processes (Needleman 2004), and genes that function in the binding and sequestration of metals (Gonick 2011). Indeed, tolerance for lead in vertebrates has been linked to metallothioneins (MT), a family of metal-binding proteins that can function in sequestering lead in a nontoxic form (Gonick 2011).

We found relatively few genes differentially expressed (DE) in high versus low lead brown anoles. In male forebrain, there were only four DE genes in high lead lizards, two upregulated and two downregulated (Figure 5A). In male liver, 51 genes were DE, with 41 upregulated and 10 downregulated (Figure 5B). In female liver, 23 genes were DE, 14 upregulated and 9 downregulated (Figure 5C).

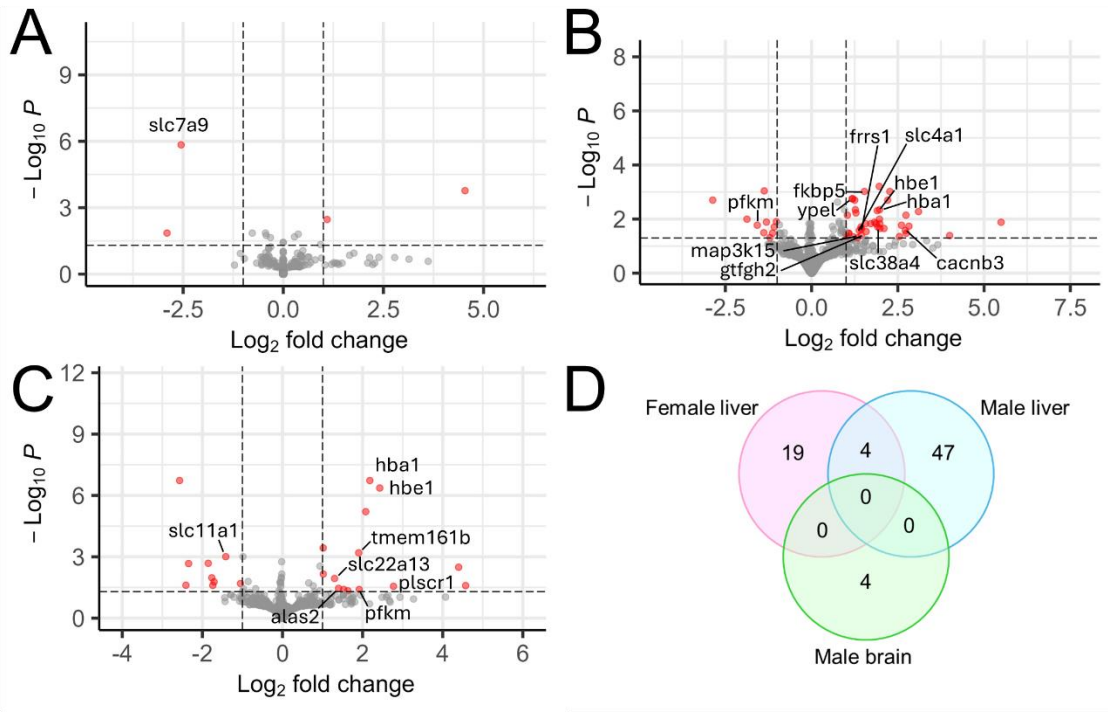

Figure 5. Differential gene expression of lizards from high compared to low lead contamination sites. Lizards were from high contamination Site F and low lead contamination Site B. Highlighted genes function in ion binding and transport, the oxidative stress response, or oxygen binding and transport. (A-C) Volcano plots of differential expression in the (A) male forebrain, (B) male liver, and (C) female liver. Cutoff for significance (red dots) is False Discovery Rate < 0.05 and an absolute log2 fold change ≥ 1. (D) Venn diagram of unique and shared differentially expressed genes across sex and tissue type.

391 There were no significantly enriched Gene Ontology (GO) categories across all DE genes
392 after correction for multiple comparisons (Table S10). However, six Biological Processes neared
393 significance (0.10 > P > 0.05), including Intracellular Iron Ion Homeostasis (GO:0006879) and
394 Oxygen Transport (GO:0015671). Many of the DE genes are involved in the binding, transport,
395 and homeostasis of metals and other ions (*alas2, frrs1, ypel3, plscr1, tmem161b, cacnb3, slc7a9,*
396 *slc4a1, slc38a4, slc22a13, slc11a1*), and are also DE under heavy metal exposure in other taxa
397 (He et al. 2009, Plusquin et al. 2012, Schneider et al. 2012, Truong et al. 2018, Smith et al.
398 2023). The response of several solute carrier genes (*slc*) is intriguing. SLCs are transmembrane
399 proteins that function in ion transport into and out of cells and have been implicated in heavy
400 metal susceptibility and tolerance (He et al. 2009, Schneider et al. 2012). We found no DE of
401 metal-binding metallothionein genes.
402 One of the key consequences of lead exposure is anemia (Fuller and Wiley 2018) because
403 lead interferes with heme production and decreases hematocrit, hemoglobin, and erythrocyte
404 levels in vertebrates (Kwong et al. 2004), including western fence lizards (Salice et al. 2009).
405 Key genes related to oxygen carrying capacity were upregulated in high lead anoles: *alas2* in
406 female liver, and *hba1* and *hbe1* in both male and female liver (two of only four genes
407 differentially expressed in both sexes). ALAS2 is a rate-limiting enzyme in heme production
408 within red blood cells (Tanimura et al. 2016). *Hba1* produces alpha globin 1, one of two globins
409 that form the mature hemoglobins that carry oxygen within vertebrate red blood cells. *Hbe1* is
410 not part of primary hemoglobin function, but *hbe1* upregulation in mice ameliorates sickle cell
411 disease and pathological underproduction of hemoglobin (thalassemia; Wang et al. 2016).
412 Furthermore, there is evidence for parallel natural selection on the *hbe1* gene in populations of
413 humans, dogs, yaks, and horses adapted to high-altitude hypoxia (Liu et al. 2019).

414 None of the top GO terms were associated with stress, and few stress-response genes
415 were upregulated in high lead lizards. There were some key exceptions. *fkbp5*, *map3k15*, and
416 *gtf2h2* are stress-related genes upregulated in male liver. *Fkbp5* produces an HSP90 cochaperone
417 integral to the stress response (Gupta et al. 2010) and is also upregulated in carp cells in response
418 to cadmium (Luan et al. 2022). *map3k15* is involved in stress-induced apoptosis, while *gtfh2* is
419 involved in DNA repair (Tulay et al. 2015, Liu et al. 2022). *pfkm*, upregulated in female liver,
420 can be induced by oxidative stress (Tang et al. 2012). None of the antioxidant genes frequently

421 associated with heavy metal exposure, such as sodium oxide dismutase, catalase, or glutathione
422 peroxidase, were DE.

423 Overall, we found minimal gene regulatory response to high levels of environmental lead
424 exposure in brown anoles. Given the lack of higher-level physiological effects (Figure 3) this is
425 perhaps not surprising, and further reinforces that these animals are very lead tolerant. Among
426 the few genes that were differentially expressed, there are intriguing clues as to how the lizards
427 tolerate lead. In particular, plasticity in the expression of solute transport genes and genes
428 involved in oxygen carrying capacity are clearly candidates for further study.

429

430 **Conclusion**

431 We found that brown anoles are reliable lead biomonitors (Burger et al. 2004) and provide
432 evidence that they have extreme lead tolerance. Indeed, they may be the most lead tolerant
433 vertebrate known. Many questions remain. Because of the scant attention paid to lizard heavy
434 metal tolerance, we do not know if our findings reflect only brown anoles in New Orleans,
435 brown anoles in general, the genus *Anolis*, or squamates more broadly (Moore et al. 2025). The
436 high lead tolerance we observed could result from evolution in response to urban lead exposure.
437 Heavy metal tolerance can evolve rapidly (Merritt and Bewick 2017), and *Anolis* lizards are
438 known to quickly physiologically evolve in response to environmental change (Gunderson and
439 Leal 2012, Campbell-Staton et al. 2017). Regardless, brown anoles represent a compelling, novel
440 vertebrate model for developing a mechanistic understanding heavy metal tolerance with
441 extensive resources available (Rasys et al. 2019, Geneva et al. 2021). Our findings also point to a
442 need for greater consideration of heavy metals in the rapidly growing field of urban ecology and
443 evolution (Rivkin et al. 2019). Cities across the globe have high levels of heavy metal pollution
444 (Clune et al. 2011), but far more effort is devoted to factors such as urban heat islands, light and
445 noise pollution, structural habitat modification, and invasive species (Rivkin et al. 2019). Taking
446 steps to better understand the effects of heavy metals in urban areas, and how they interact with
447 other well-known urban stressors, outside of a traditional ecotoxicological context will likely
448 yield important insights (Schneeweiss et al. 2023, Sigmund et al. 2023).

449

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Competing Interests

The authors declare no competing interests.

Author Contributions

A.B., A.G. and J.K. conceived the project. A.B. and A.J.F. conducted physiological experiments. A.B. and K.S. conducted bioinformatic analyses. A.B. and A.G. wrote the paper with input from other authors.

Data Availability

Supplementary information is available for this paper. Data presented here are available in a GitHub repository (https://github.com/AnolisNOLA/Brown-anole-data)

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