Using *Peromyscus leucopus* as a biomonitor to determine the impact of heavy metal exposure on the kidney and bone mineral density: Results from the Tar Creek Superfund Site

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Background. Human population growth and industrialization contribute to increased pollution of wildlife habitats. Heavy metal exposure from industrial and environmental sources is still a threat to public health, increasing disease susceptibility. In this study, I investigated the effects of heavy metals (cadmium (Cd), lead (Pb), and zinc (Zn)) on kidney and bone density. Objective. This study aims to determine the concentrations of Cd, Pb, and Zn in soil and compare them to the levels of the same metals in *Peromyscus leucopus* kidney tissue. Furthermore, the study seeks to investigate the impact of heavy metals on bone density and fragility using the fourth lumbar vertebra (L4) of *P. leucopus*.

Methods. Cd, Pb, and Zn concentrations in soil specimens collected from Tar Creek Superfund Site (TCSFS), Beaver Creek (BC), and two reference sites (Oologah Wildlife Management Area [OWMA] and Sequoyah National Wildlife Refuge [SNWR]). Heavy metal concentrations were analyzed using inductively coupled plasma-mass spectroscopy (ICP-MS). Micro-computed tomography (μCT) was used to assess the influence of heavy metals on bone fragility and density.

Results. On the one hand, soil samples revealed that Pb is the most common pollutant in the sediment at all of the investigated sites (the highest contaminated site with Pb was TSCF). Pb levels in the soil of TCSFS, BC, OWMA, and SNWR were found to be 1132±278, 6.4±1.1, and 2.3 ±0.3 mg/kg in the soil of TCSFS, BC and OWMA and SNWR, respectively. This is consistent with the fact that Pb is one of the less mobile heavy metals, causing its compounds to persist in soils and sediments and being barely influenced by microbial decomposition. On the other hand, the kidney samples revealed greater Cd levels, even higher than those found in the soil samples from the OWMA and SNWR sites. Cd concentrations in the kidney specimens were found to be 4.62±0.71, 0.53±0.08, and 0.53±0.06 μg/ kg, respectively. In addition, micro-CT analysis of L4 from TCSFS showed significant Pearson's correlation coefficients between Cd concentrations and trabecular bone number (-0.67, p≤ 0.05) and trabecular separation (0.72, p≤ 0.05). The results showed no correlation between bone parameters and metal concentrations at reference sites. This study is one of the few that aims to employ bone architecture as an endpoint in the field of biomonitoring. Furthermore, this study confirmed some earlier research by demonstrating substantial levels of heavy metal contamination in soil samples, kidney samples, and *P. leucopus* L4 trabecular bone separations from TCSFS. Moreover, this is the first study to record information regarding bone microarchitecture parameters in *P. leucopus* in North America.

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Abstract

Background. Human population growth and industrialization contribute to increased pollution of wildlife habitats. Heavy metal exposure from industrial and environmental sources is still a threat to public health, increasing disease susceptibility. In this study, I investigated the effects of heavy metals (cadmium (Cd), lead (Pb), and zinc (Zn)) on kidney and bone density. Objective. This study aims to determine the concentrations of Cd, Pb, and Zn in soil and compare them to the levels of the same metals in *Peromyscus leucopus* kidney tissue. Furthermore, the study seeks to investigate the impact of heavy metals on bone density and fragility using the fourth lumbar vertebra (L4) of *P. leucopus*.

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42 from the OWMA and SNWR sites. Cd concentrations in the kidney specimens were found to be
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51 to record information regarding bone microarchitecture parameters in *P. leucopus* in
52 North America.
53 Keywords.
54 Tar Creek Superfund site, Heavy metals, *Peromyscus leucopus*, Bone fragility,
55 Microarchitecture, Biominitors*.*
56

Introduction

58 Tar Creek Superfund site (TCSFS), Ottawa, OK is in northeastern Oklahoma near the
59 Kansas-Oklahoma border. TCSFS covers a 40-square-mile area and is one of the Tri-
60 State Mining District (Oklahoma, Kansas, and Missouri) sites (Bigby & Jim 2021; Coffin
61 et al. 2022; Hayhow 2021; McCann & Nairn 2022; Park et al. 2020). These sites include
62 territories of ten tribal nations such as the Quapaw Nation and several other
63 communities such as Picher, Cardin, North Miami, and Commerce (Agency for Toxic
64 Substances and Disease Registry, 2015). Lead and zinc ores were mined at TCSFS
65 from the early 1900s to the late 1970s.
66 *Peromyscus. leucopus* has been the subject of several different studies on the effects of
67 environmental contaminants (Beyer et al. 2018; Johnson et al. 2021; Rubino et al.
68 2021).
69 The Agency for Toxic Substances and Disease Registry (ATSDR) lists the major
70 pathways of exposure to lead contamination at TCSFS as contaminated air,
71 contaminated water, contaminated food resources, and contaminated soil. Such human
72 health problems as respiratory illness, liver dysfunction, and reproductive and renal
73 failure can occur after exposure via these pathways (ATSDR, 2015; Briffa et al. 2020).
74 Heavy metals such as cadmium, lead, and zinc at TCSFS were studied because of their
75 potential effects on human health and their accumulation in small mammals' bodies and
76 other terrestrial fauna (Briffa et al. 2020; Martín et al. 2021; Monchanin et al. 2021;
77 Sánchez-Chardi & Nadal 2007). Numerous human health issues have been
78 documented after exposure to cadmium because of its ability to substitute for other
79 metals and nutrients such as zinc (Beyersmann & Hartwig, 2008; Briffa et al. 2020).
80 Depending on the concentration, duration, and species of the metals involved, exposure
81 to metals has a detrimental effect on bone health and may lead to osteoporosis and an

82 increased risk of fracture. Lead and certain other metals are stored permanently in
83 bones, which in mammals and birds may hold 90% of the total body burden (Bjørklund
84 et al. 2020). Furthermore, lead, cadmium, and chromium may have a non-redundant
85 function in the pathogenesis of osteoporosis at the cellular/molecular and epigenetic
86 levels (Scimeca et al. 2017).
87 The most common contaminants in the soil are cadmium as a heavy metal and atrazine
88 as an organic pollutant, both can be considered potential pathogenic pollutants (Said et
89 al. 2022; Scimeca et al. 2017). Small amounts of free cadmium ions are more toxic than
90 bound cadmium ions, and cadmium can cause toxicity in different organs including the
91 pancreas, testis, and nervous system. Elimination of cadmium by the kidneys is slow.
92 Chronic exposures to cadmium ions can result in proteinuria and tubular dysfunction in
93 the proximal tubules (Davenport 2020; Godt et al., 2006; Yan & Allen 2021; Zavala-
94 Guevara et al. 2021). Renal toxicity from cadmium exposure is correlated with the
95 number of cadmium ions in kidney tubule cells, reabsorption, degradation of cadmium
96 metallothionein complexes, and excess production of metallothionein by renal tubules
97 (Davenport 2020; Zavala-Guevara et al. 2021). Cadmium toxicity observed in the renal
98 cortex of laboratory rats (*Rattus rattus*) resulted in cytosolic damage and renal
99 malfunction after oral administration of cadmium chloride ($CdCl_2$) (Alimba et al. 2021;
100 Hashim et al. 2018; Siddiqui, 2010).
101 Lead is a non-essential metal and is one of the abundant toxic metals at TCSFS.
102 Lead exposure can result in acute and chronic toxic effects. Lead accumulates in wild
103 mammals in tissues such as the kidney, liver, and bone. In smelter and mining sites,
104 lead and zinc were recorded in wood mice (*A. sylvaticus*), bank voles (*C. glareolus*),
105 and field voles (*M. agrestis*), with high lead concentrations in bones; 42-68% of total
106 lead found in body tissues was contained in bone (Bjørklund et al. 2020; Ecke et al.
107 2020; Jasiulionis et al. 2018; Turna Demir & Yavuz 2020).
108 Lead exposure decreases bone mineral density (BMD) which can cause osteoporosis
109 (Bjørklund et al. 2020; Campbell et al., 2004; Scimeca et al. 2017). In addition, lead
110 exposure inhibits osteoblast function. Zinc is another trace metal that was measured in
111 this study. Zinc has important functions in bone formation, turnover, and metabolism
112 (Bekheirnia et al., 2004; Ceylan et al. 2021; Gaffney-Stomberg 2019). Zinc as a cofactor
113 plays essential roles in other tissue and enzyme functions that are important for bone
114 mineralization and development such as alkaline phosphate and collagenase
115 (Bekheirnia et al., 2004; Ceylan et al. 2021; Gaffney-Stomberg 2019).
116 Bone as a connective tissue has different sizes, shapes, and structures that serve
117 important functions. Mineralized bone is the osseous tissue that gives bone rigidity.
118 Bone tissue accumulates heavy metals such as cadmium and lead. Furthermore, bone
119 tissue is one of the tissue markers that indicate xenobiotic and metal exposure
120 (Bjørklund et al. 2020; Scimeca et al. 2017). *P. leucopus* as bioindicator species
121 recorded as the most common species of the small mammals at TCSFS is the white-

122   footed mouse (*Peromyscus leucopus*) (Park et al. 2020; Phelps & McBee, 2009; Phelps
123   & McBee, 2010).
124   Studying bone microarchitecture helps to evaluate the toxic effects on bone after
125   exposure to heavy metals. Bone dysfunction and osteoporosis are reported as toxic
126   effects of exposure to cadmium (Buha et al. 2019; Luo et al. 2021; Youness et al.,
127   2012). A significant decrease in bone density and the presence of osteopenia has been
128   recorded in women who are exposed to cadmium from environmental sources
129   (Engström et al., 2012; (Jalili et al. 2020; Kim et al. 2021).
130   Because soil is a valuable natural resource that may contain environmental
131   contaminants, the concentrations of heavy metals (cadmium (Cd), lead (Pb), and zinc
132   (Zn) in TCSFS, BC, and reference sites were determined using soil samples. The
133   presence of contaminants in a biological source is assessed using soil, and changes in
134   the physiological parameters of *P. leucopus* as a biomonitoring species are
135   investigated. As a result, the goal of this study is to compare the levels of Cd, Pb, and
136   Zn in the soil to those found in *Peromyscus leucopus* kidney tissue. The study uses the
137   fourth lumbar vertebra (L4) of *P. leucopus* to investigate the effect of heavy metals on
138   bone density and fragility. As a result, we may have a better understanding of how these
139   contaminants affect the natural resources of the environment as well as the associated
140   fauna. Furthermore, one of the purposes of the study is to link biological factors to the
141   presence of heavy metals in soil in a field setting. This study is one of the few that aims
142   to employ bone architecture as an endpoint in the field of biomonitoring. Moreover, this
143   is the first study to record information regarding bone microarchitecture parameters in *P.*
144   *leucopus* in North America.
145

Materials & Methods

Study sites

148   The study included three different sites. Tar Creek Super Fund Site (TCSFS) at Beaver
149   Creek and two reference sites, Sequoyah National Wildlife Refuge (SNWR) and
150   Oologah Wildlife Management Area (OWMA) (Figure.1). Soil samples were collected
151   from these sites for metal analysis (lead, cadmium, and zinc). The TCSFS has a large
152   population of white-footed mice (*Peromyscus leucopus*) which were used in this study to
153   analyze the influence of heavy metals on the physiological alteration in kidney tissues.
154   Both SNWR and OWMA are good sites to compare with TCSFS because no mining has
155   been recorded on these sites. The use of two reference sites rather than just one
156   provides a more accurate comparison to the highly contaminated TCSFS. In addition,
157   these two sites are geographically distant (Figure.1). Other reasons to choose these
158   sites were to compare relationships among soil metal levels, tissue metal levels, and
159   metallothionein induction in *P. leucopus* species, which is present in all three
160   sites.Position for collection sites was detected with the help of Eterx Vista CX Garmin
161   and Google. Samples were collected to a depth of 18-20 cm from each position

162 (Figure.1). Field experiments were approved by the Oklahoma State University
163 Institutional Animal Care and Use Committee (ACUP No. AS 066).

164 **Soil Sampling**
165 Soil samples were collected from the study sites following the procedure that is
166 described by USEPA (United States Environmental Protection Agency, 2005). A
167 random design was used to collect soil samples in each site separately. Moreover, a
168 meter scale was used to measure the distances between soil samples. Eight duplicate
169 soil samples were collected from each site. Each sample was labeled as T1-1, T1-2, T2-
170 1, T2-2, etc. where T identifies TCSFS, the first numeral (1-8) indicates the number of
171 the sample, and the second numeral (1-2) points to the original or the duplicate.
172 Samples were collected from each position with a 10% HCl acid–washed metal scoop
173 to a depth of 18-20 cm. Samples were homogenized and weighed separately. Soil
174 samples were dried in separate 10% HCl acid-washed and labeled polypropylene plates
175 at room temperature for two weeks. Also, dried soil samples were sieved twice using
176 the first 1mm sieve size, No. 18 (USA. Standard Test Sieve) followed by a second 250
177 μm opening sieve (Fisher Scientific Company, Pittsburgh, Pennsylvania). Samples were
178 saved in labeled acid-washed glass bottles.

179 **Soil digestion**
180 Soil samples were prepared in the trace mineral laboratory of the Nutrition Sciences
181 Department using microwave digestion. All soil samples from Tar Creek Super Fund
182 Site (TCSFS) and the two reference sites, Sequoyah National Wildlife Refuge (SNWR)
183 and Oologah Wildlife Management Area (OWMA) were weighed separately using the
184 same digital balance and labeled polypropylene plates. Soil samples were digested
185 using microwave digestion protocol (Milestone, Inc, Shelton, Connecticut) specified by
186 USEPA Method Number 3051A (Kingston et al., 1997; United State Environmental
187 Protection Agency, 1986) according to the EPA procedure. Following the protocol for
188 microwave digestion, all soil sample sizes were 0.5 g. For all soil samples, 0.5 g of soil
189 was put in the labeled microwave vessel. Double distilled trace element grade nitric acid
190 (99.999%) was purchased from Fisher Scientific Company (Pittsburgh, Pennsylvania).
191 Microwave vessels were transferred to the hood and 10 ml of HNO3 was added to the
192 soil sample in each vessel. The soil and acid solution was swirled slowly to mix soil with
193 acid. A Teflon cover was placed on the Teflon vessel and pushed down, and an adaptor
194 was placed on the flat part of the Teflon cover. The Teflon indicator ring was placed on
195 the cover and pushed shut. Vessels were then introduced into the polypropylene
196 microwave rotor. Each indicator was closed tightly with a torque wrench. A
197 thermocouple was placed into reference vessel No.1 that contained a blank laboratory
198 (control) sample, the microwave door was closed, and the machine was switched on.
199 The program was set on the USEPA Method Number 5031A. Samples were heated for
200 50 minutes at 100° C. Next, the segments were pulled gently from the microwave after 5
201 minutes because they were hot and opened slowly using a torque wrench to release the

202 pressure from the vessels. Samples were transferred to the hood to open the vessel's
203 cover and yellow acid fumes evaporated from the vessels. The acid solution from
204 vessels 10 ml was poured into a plastic 15 ml tube purchased from VWR. Vessels were
205 washed with 5 ml double distilled water, and water was added to the first tube. All
206 samples were digested following the same process. The digested soil samples were
207 centrifuged at 1200 g for 10 minutes. The samples were decanted into a new tube (2nd
208 tube) gently and slowly to avoid solution contamination. The first tube that contained the
209 soil was disposed of separately. The second tube for each digested sample was labeled
210 as stock that was used for metal analysis. 0.2 ml of the digested soil solution in 10 ml
211 total volume samples were diluted by adding double distilled water (DDW) to 0.20 ml
212 sample solution in the new tube (3rd tube). This tube of each sample was used for the
213 Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) instrument (Perkin Elmer) to
214 analyze metal (Pb, Zn, and Cd) concentrations in each sample separately.

215 **ICP-MS analysis**
216 Cd, Pb, and Zn concentrations in soil specimens from both contaminated and
217 uncontaminated sites were determined by ICP-MS according to USEPA Method 6010
218 (United States Environmental Protection Agency, 1996). Terbium was used as an
219 internal standard (Perkin Elmer, Shelton, CT). The ICP-MS instrument was calibrated to
220 ensure the stability and consistency of all results. Before running samples by ICP-MS,
221 five standard dilutions for ICP-MS were diluted. Internal standard (Terbium) solution
222 (Perkin Elmer, Shelton, Connecticut) and double distilled water (DDW) were used for
223 calibration and dilutions. All sample volumes were run in ICP-MS as 0.1 µl sample
224 diluted to 10 ml (DDW) and add 20 µl (IS). Sample tubes were vortexed for 20 seconds
225 before analysis.

226 **Kidney sampling**
227 Frozen kidney biopsies of *Peromyscus leucopus* were provided by the OSU Collection
228 of Vertebrates, Department of Integrative Biology. The *P. leucopus* had been collected
229 from TCSFS, BC, and the reference sites, OWMA and SNWR. The kidneys were
230 already frozen at -80° C. Kidney samples were saved in a liquid nitrogen tank before
231 subsampling. The kidneys were cut in a plastic petri dish placed on wet ice using a
232 stainless-steel scalpel to one-tenth of the whole tissue and saved in plastic microtubes.
233 Next, kidney samples were refrigerated at -40° C prior to digestion for metal analysis.
234 Oklahoma State University Animal Care and Use provided full approval for this research
235 (Protocol AS056).

236 **Tissue digestion**
237 Kidney samples (Kid) were weighed using a digital balance. Small microwave tubes
238 were used for kidney sample digestion (Milestone, Inc, Shelton, Connecticut) and
239 USEPA Method Number 3051A (United State Environmental Protection Agency, 1986).
240 Each sample was weighed and put in small microwave tubes; 1 ml of concentrated
241 (99.999) HNO_3 was put in the tube and then 0.15 ml of H_2O_2 was added to the same

242   tube. The program was set on USEPA Method Number 5031A. The microwave
243   operation required 50 minutes to finish digestion at the temperature of 100° C. Next, the
244   vessels were pulled gently from the microwave after 5 minutes. The segments were
245   opened slowly using a torque wrench to release the pressure from the vessels. Samples
246   were transferred to the hood to open the vessel's cover, and yellow acid evaporated
247   from the vessels.

248   **Metal analysis**

249   To ensure the stability and consistency of all results, Cd, Pb, and Zn concentrations in
250   kidney specimens were determined by inductively coupled plasma mass spectrometry
251   (ICP-MS) instrument (Perkin Elmer) according to USEPA method 6010 (United States
252   Environmental Protection Agency, 1996). Terbium was used as an internal standard
253   (Perkin Elmer, Shelton, CT). Before samples were run, five standard dilutions for ICP-
254   MS were prepared. All sample volumes were analyzed by ICP-MS as 80 µl sample with
255   10 µl Internal standard (Terbium) solution (Perkin Elmer, Shelton, Connecticut) and
256   complete volume by DDW up to 5 ml. Sample tubes were vortexed for 20 seconds
257   before analysis by ICP-MS.

258   **Bone microarchitecture:**

259   **µCT analysis:**

260   Eight skeletons of *P. leucopus* from each site were provided from the vertebrate
261   collection from the Department of Integrative Biology at OSU. The lumbar 2, 3, 4, and 5
262   vertebrae section of each skeleton was excised and the L4 was scanned using a high-
263   resolution computed tomography system or micro-CT scanner (µCT 40, Scano Medical
264   AG, Zurich, Switzerland). The fourth lumbar vertebra was detected in each skeleton
265   sample and saved as a 3-D image. The trabecular bone in the 3-D images of L4 was
266   contoured in a 300-400 µCT slide image. L4 slices were contoured every 10 slices
267   beginning 10 slices below the detection of the spongiosa and ending 10 slices from the
268   growth plate. The threshold for evaluation was set as 350 (grayscale, zero-1000) for all
269   slides. The trabecular bone was contoured to measure the connectivity density
270   (ConnD), trabecular separation (TbSp), trabecular thickness (TbTh) (mm), trabecular
271   number (TbN) (mm-1), and trabecular volume (TV) as a percent of bone volume fraction
272   (BVTV) (bone volume/tissue volume) for individual lumbar vertebra. The 3D images of
273   the results were evaluated, and the data set was exported to evaluate and analyze the
274   results.

275   **Statistical analyses**

276   This study examined soil and kidney samples collected from a contaminated site,
277   TCSFS, BC, and compared them with reference sites (SNWR and OWMA). In addition
278   to soil and kidney samples, bone parameters from the contaminated site were
279   compared with reference sites. Metal concentrations in the kidney were correlated with
280   bone parameters. Pearson's correlation coefficients were determined for all samples

281 taken together and by individual sites; Proc GLM, Proc Corr, SAS, V 9,4 were used and
282 values of P<0.05 were taken as significant.

Results

Soil heavy metal levels:

285 Comparison of the heavy metal levels in the soil samples from the contaminated site
286 (TCSFS, BC), and the reference sites SNWR and OWMA (Tables 1-5) showed that
287 cadmium concentrations in the soil samples of TCSFS, BC, and the reference sites
288 were sharply different. Mean concentrations of Cd, Pb, and Zn mg/kg in soil samples
289 are presented in Table 1. In TCSFS, BC as a contaminated site, soil cadmium
290 concentrations (mean±SE) at 48±04 mg/kg were recorded significantly higher than
291 those at the two reference sites (0.06±0.01, and 0.15±0.03 mg/kg).
292 As expected, lead concentrations in the TCSFS, BC soil samples (1132±278 mg/kg)
293 were higher (P<0.0001) than in the two reference sites (2.3±0.33, 6.4±1.1 mg/kg). Zinc
294 concentrations in TCSFS, BC (14083±1826) were also much higher (P<0.0001) than in
295 the two reference sites (20±2 and 53±5 respectively) (Table.1).

Kidney heavy metal levels

297 As for the kidney samples (µg/ Kg), the zinc concentrations showed no significant
298 differences between the contaminated TCSFS, BC, and the reference sites OWMA and
299 SNWR. Metal concentrations (Cd, Pb, and Zn) in kidney samples (µg/ Kg) are
300 presented by the site in Table 2.
301 Cadmium concentrations (µg/ Kg) in kidney samples from TCSFS, BC and reference
302 sites were significantly different (Table 2). In TCSFS, BC, higher cadmium
303 concentrations (4.62±0.71 µg/ Kg) were recorded than at the two reference sites
304 (P≤0.0005), but the results showed no differences between the two reference sites,
305 OWMA (0.53 ±0.10) and SNWR (0.53 ±0.06) µg/ Kg. Likewise, Pb concentrations in
306 kidney samples from TCSFS, BC (0.57±0.10 µg/ Kg) were higher than in the two
307 reference sites, OWMA and SNWR (0.04±0.01 and 0.05±0.01 µg/ Kg respectively).

Bone microarchitecture relation to heavy metals at TCSFS, BC site

309 In TCSFS, BC, Cd concentration was positively correlated with trabecular bone
310 separation (r=0.72, P ≤ 0.05). Correlation between bone parameters and kidney mineral
311 concentrations by individual site and Pearson's correlation coefficients are presented in
312 Table 4. Cadmium concentrations were negatively correlated with the trabecular bone
313 number (r=-0.67, P ≤ 0.05), and Pb concentration was positively correlated with
314 trabecular bone separation (r=0.72, P ≤ 0.05).

Bone microarchitecture parameters in relation to heavy metals for uncontaminated sites

316 Trabecular bone microarchitecture parameters for the lumbar vertebrae (L4) and kidney
317 metal concentrations (Cd, Pb, and Zn) in *Peromyscus leucopus* revealed 1) the
318 differences between the contaminated site TCSFS, BC site (Table 4) and 2) the and the
319 reference sites (n=16) (Table 5). Micro-computed tomography evaluation results of bone

320 parameters showed no correlations with kidney Cd, Pb, and Zn. Kidney lead positively
321 correlated with kidney Zn (0.85, ≤ 0.05).

Discussion

323 This environmental toxicology field study showed several impacts and physiological
324 alterations in *Peromyscus leucopus* due to their contact with the contaminants Cd, Pb,
325 and Zn (Tables 1-5). The present study used specimens collected from the TCSFS, BC
326 contaminated area, and two reference sites (OWMA and SNWR). As expected, the
327 concentrations of heavy metals (Cd, Pb, and Zn) in soil and kidney at TCSFS, BC were
328 higher than at the reference sites. This study also analyzed the correlations between
329 mineral concentrations (Cd, Pb, and Zn) of the kidney and the biomarkers such as bone
330 parameters in the biomonitoring species *Peromyscus leucopus*.
331 Several studies have determined that TCSFS is a site highly contaminated with Cd, Pb,
332 and Zn. Mineral analysis of soil sample results confirmed heavy metal contamination at
333 TCSFS, BC compared to reference sites (OWMA and SNWR). recorded the elevation
334 of cadmium, lead, and zinc in soil sediments at Beaver Creek and Douthat Settling
335 Pond at TCSFS. Lead concentrations were 440-540 mg/kg and cadmium
336 concentrations were 20-56 mg/kg while zinc concentrations were 3000-9300 mg/kg.
337 Large amounts of chat at TCSFS and extensive amounts of Cd, Pb, and Zn from mining
338 and acid water were reported from the 1900s through the 1960s (Oklahoma Department
339 of Environmental Quality, 2003). Heavy metals Cd, Pb, and Zn in tailings and yard soil
340 at the Tar Creek National Priorities List Superfund site in Oklahoma were analyzed in
341 order to reduce metal and restore vegetation in this area (Brown et al., 2007). Soil
342 chemical analysis showed unequal and extended distribution of heavy metals at the Tar
343 Creek superfund site at chat near Pitcher Oklahoma. The highest concentration of the
344 contaminants was zinc > 4000 ppm, lead >1000ppm, and cadmium > 40 ppm (Beattie et
345 al., 2017).
346 Bone is one of the most targeted tissues by lead (Al-Ghafari et al. 2019; Lerner et al.
347 2019; Yang et al. 2019). Lead toxicity effects on bone cellular levels cause alterations.
348 These effects include changes in circulating hormone 1, 25-dihydroxy vitamin D3 which
349 regulates bone functions (Al-Ghafari et al. 2019; Lerner et al. 2019). Also, Martiniaková
350 et al. (2010) recorded significant heavy metal concentrations in *Apodemus flavicollis*
351 and *Apodemus sylvaticus* at another polluted site in Slovakia. Although slight heavy
352 metal accumulations were recorded in the femora, the study observed no changes in
353 the femora's bone weight and the length of both species.
354 The present study affirms previous studies which have documented major effects on
355 bone density and osteoporosis resulting from cadmium and lead exposure, including
356 humans (Youness et al., 2012). In addition, the findings of this study are also consistent
357 with other studies which have shown that heavy metals can cause liver and renal
358 damage. For example, Lavery et al. (2009) investigated heavy metal effects on bone
359 density, other bone parameters, renal damage, and metallothionein (MT) concentrations

360   of South Australian bottlenose dolphins (*Tursiops aduncus*). The results showed Cd, Zn,
361   and Cu in *Tursiops aduncus* caused liver as well as renal damage. Bone parameters of
362   two individuals of *Tursiops aduncus* showed dysfunctions, renal damage, and high
363   levels of MT (Lavery et al., 2009).
364   The findings of this study regarding cadmium contamination at TCSFS, BC have
365   important implications for human health. Bone resorption and negative health effects
366   have been shown to increase in women after middle age due to exposure to even low
367   levels of cadmium in the diet (Åkesson et al., 2006). According to a study of women in
368   southeast China in an area heavily polluted by cadmium, cadmium affected bone
369   formation and turnover through indirect effects on vitamin D3 metabolism (Wang et al.,
370   2003). Heavy metal toxicity reduces the function of micro and macronutrients such as
371   Zn, phosphate, and calcium which are the main components for bone strength and
372   density.
373   Generally, this study recorded higher metal concentrations in soil and kidney samples
374   from TCSFS compared with two reference sites. However, the bone microarchitecture
375   analyses of *Peromyscus leucopus* L4 vertebra of contaminated and uncontaminated
376   sites did not show a strong correlation between bone parameters and metal
377   concentration in the kidney. The lack of significant differences could have resulted from
378   a limited sample size (n=8 from each site). Correlations between bone microarchitecture
379   variables appeared to be higher in TCSFS, and BC samples than in reference sites.
380   In addition, this study used only adult mice, which are more exposed to the
381   contaminants than younger mice due to their age. However, we did not know the exact
382   age of the mice in this study. Bone mineral density and other bone parameter changes
383   can be influenced by age. Legrand et al. (2000) recorded several vertebra fractures in a
384   male patient of 52 years of age with lumbar osteopenia. These findings are evaluated
385   through X-ray absorptiometry and bone microarchitecture changes of L2 and L4
386   trabecular bone. Bone resorption is associated with the inhibition of osteoblast function,
387   and the studies reported this inhibition associated with the lead effects on cellular
388   functions and regulation such as 1, 25–dihydroxy vitamin D3 (Lerner et al. 2019; Yang
389   et al. 2019)). Variations in bone parameters were expected due to the specimens'
390   habitat, environmental contaminants, and variable ages.
391   In the current study, the contaminated site (TCSFS) showed significant elevation in
392   metal concentrations in soil, kidney, and L4 trabecular bone separations of *P. leucopus*.
393   This study showed metal accumulation in a small mammal, *P. leucopus*. The analysis of
394   heavy metal concentrations in the kidney may elucidate issues of environmental quality
395   and physiological alteration.
396
397   **Conclusions**
398

399 The results of this study reflected actual environmental contamination, but due to the
400 field conditions, this study had several uncontrolled variables such as contaminant
401 levels, duration of exposure to the heavy metals, and animal age. The contaminated site
402 TCSFS, BC location was far from reference sites OWMA & SNWR (SNWR is located
403 substantially further than OWMA from TCSFS). The results are a very important
404 approach to determining the specific endpoint of concern and reaching conclusions that
405 help human health and environmental sustainability. The fact that these mice, *P.
406 leucopus*, had such high mineral concentrations in their kidneys and were still alive
407 raises the possibility that this species, *P. leucopus* adapted to the heavy metal
408 exposure. Moreover, this is the first study to record information regarding bone
409 microarchitecture parameters in *P. leucopus* in North America.
410

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420

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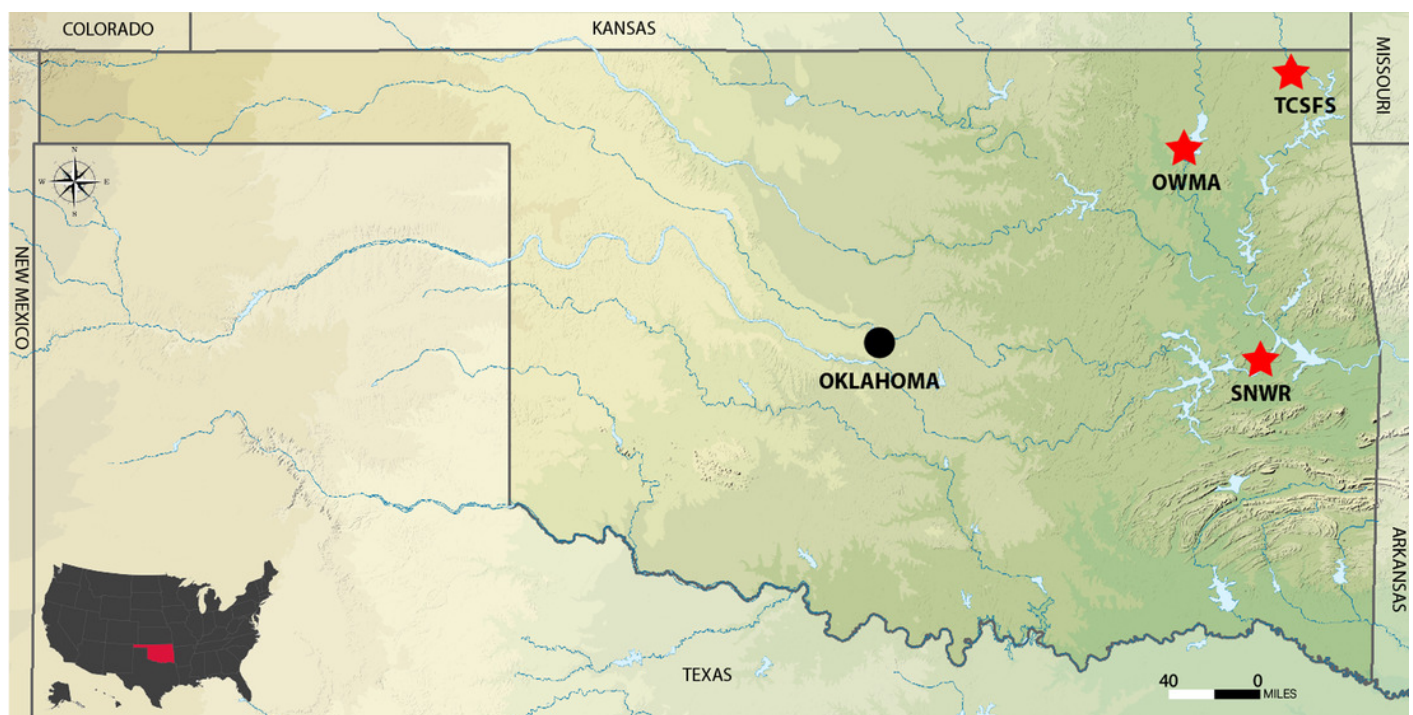
Figure 1

Location of the Tar Creek Superfund Site and two reference study areas (red stars) in northeastern Oklahoma State, USA.

__OWLA__, Oolaga wild life management area. __SNWR__, Sequoyah national wildlife refuge. __TCSFS__, Tar Creek Superfund Site. Adapted from Wikimedia Commons (https://upload.wikimedia.org/wikipedia/commons/1/16/USA_Oklahoma_relief_location_map.svg) by SANtosito, 3 February 2021. Creative Commons Attribution-Share Alike 4.0 International license.

Concentrations of zinc, cadmium, and lead in soil samples gathered from the study areas.

OWMA, *Oologah Wildlife Management Area.* ***SNWR***, *Sequoyah National Wildlife Refuge.* ***TCSFS***, *Tar Creek Superfund site. Means in rows not sharing the same superscript are significantly different from each other (P<0.0001).*

Mineral /Site	n	SNWR	OWMA	TCSFS
Zn (mg/kg)	16	20.0±1.9 [b]	52.6±5.0 [b]	14083.9±1825.8[a]
Cd (mg/kg)	16	0.06±0.01[b]	0.15 ±0.03 [b]	48.04±3.98 [a]
Pb (mg/kg)	16	2.3 ±0.3 [b]	6.4±1.1 [b]	1132 ±278 [a]

Body weights and kidney lead, cadmium, and zinc concentrations of Peromyscus leucopus individuals gathered from study areas.

BW, *body weight.* **OWMA**, *Oologah Wildlife Management Area.* **SNWR**, *Sequoyah National Wildlife Refuge.* **TCSFS**, *Tar Creek Superfund site. Means in rows not sharing the same superscript are significantly different from each other (P<0.0001).*

Variables (Mean ±SE)	TCSFS	OWMA	SNWR
BW (g)	25 ± 0.8^a	$24\pm1.3a$	23 ± 1.2^a
Kidney Pb (µg/Kg)	0.57 ± 0.10^a	0.04 ± 0.01^b	0.50 ± 0.01^b
Kidney Cd (µg/Kg)	4.62 ± 0.71^a	0.53 ± 0.08^b	0.53 ± 0.06^b
Kidney Zn (µg/Kg)	23.1 ± 3.3^a	18.5 ± 3.8^a	28.4 ± 4.6^a
n	17	16	16

1

Bone microarchitecture parameters of Peromyscus leucopus (n=24) collected from the study sites.

BVTV, *Bone Volume/Total Volume (bone volume fraction).* **ConnD**, *Connectivity Density.* **OWMA**, *Oologah Wildlife Management Area.* **SNWR**, *Sequoyah National Wildlife Refuge.* **TbN**, *Trabecular Number.* **TbSp**, *Trabecular separation.* **TCSFS**, *Tar Creek Superfund Site.* **TV**, *Total Volume.* Means in rows not sharing the same superscript are significantly different from each other (P<0.0001).

Variables (mean±SE)	TCSFS	OWMA	SNWR
TV	1.27±0.14 [a]	0.81±0.06 [b]	0.10±0.12 [b]
BVTV	0.16±0.02	0.18±0.01	0.15±0.02
ConnD	238.16±50.34 [a]	157.41±12.55 [b]	132.81±16.75 [b]
TbN	4.11±0.38	4.07±0.11	3.63±0.12
TbSp	0.26±0.03	0.24±0.01	0.28±0.02

Pearson's correlation coefficients for trabecular bone microarchitecture parameters of L4 and kidney metal concentrations from Peromyscus leucopus (n=8) collected from the Tar Creek Superfund Site (TCSFS).

BVTV, Bone Volume/Total Volume (bone volume fraction). ConnD, Connectivity Density. Kid, Kidney. TbN, Trabecular Number. TbSp, Trabecular separation.TbTh,Trabecular thickness. P≤0.05*, P≤0.005**, P≤0.0005***.

Variables	1	2	3	4	5	6	7	8
BVTV	—	—	—	—	—	—	—	—
ConnD	0.81*	—	—	—	—	—	—	—
TbN	0.87**	0.95***	—	—	—	—	—	—
TbTh	0.19	-0.31	-0.28	—	—	—	—	—
TbSp	-0.82*	-0.89**	-0.98***	0.31	—	—	—	—
Kid-Cd	-0.49	-0.52	-0.67*	0.21	0.72*	—	—	—
Kid-Pb	0.58	0.21	0.41	0.46	-0.51	-0.43	—	—
Kid-Zn	-0.24	-0.21	0.01	-0.47	-0.14	-0.13	0.22	—

Pearson's Correlation coeffic i ents for trabecular bone microarchitecture parameters of L4 and kidney metal concentrations from *Peromyscus leucopus* (n=16) collected from Oologah Wildlife Management Area and Sequoyah National Wildlife Refuge.

BVTV, *Bone Volume/Total Volume (bone volume fraction).* *ConnD*, *Connectivity Density.* *Kid*, *Kidney.* *TbN*, *Trabecular Number.* *TbSp*, *Trabecular separation.* *TbTh*, *Trabecular thickness.* *P≤0.05*, P≤0.005**, P≤0.0005***.*

Variable	1	2	3	4	5	6	7	8
BVTV	0.02	_	_	_	_	_	_	_
ConnD	- 0.14	0.37	_	_	_	_	_	_
TbSp	0.17	-0.52*	-0.83***	_	_	_	_	_
TbN	-0.29	0.56**	0.86***	-0.97***	_	_	_	_
Kid-Cd	-0.22	0.26	0.08	-0.30	0.33	_	_	_
Kid-Pb	-0.20	-0.17	0.09	-0.20	0.19	0.37	_	_
Kid-Zn	0.11	-0.20	-0.02	-0.17	0.11	0.39	0.85***	_