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Assessing Metallothionein 1 Response and Heavy Metal Concentrations in Peromyscus Leucopus at the Tar Creek Superfund Site, USA

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ABSTRACT

Heavy metal contamination is a critical environmental issue, particularly in areas with a history of industrial activities such as mining. Here, we examine the concentrations of lead, cadmium, and zinc, as well as Metallothionein-1 (MT-1) expression, in white-footed mice (Peromyscus leucopus) from the Tar Creek Superfund Site (TCSFS), and two reference sites: Oologah Wildlife Management Area and Sequoyah National Wildlife Refuge, Oklahoma, USA. Using inductively coupled plasma-mass spectrometry (ICP-MS) and enzyme-linked immunosorbent assays (ELISA), we quantified metal concentrations and MT-1 levels in kidney tissues. Pb (0.57 ± 0.10 µg/kg) and Cd (4.62 ± 0.71 µg/kg) concentrations were significantly higher at TCSFS, while Zn levels (23.1 ± 3.3 µg/kg) were consistent across sites. MT-1 expression showed no significant differences, indicating a complex response to metal exposure. These findings highlight the need to further investigate MT-1 regulation and its potential as a biomarker for metal toxicity.

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Introduction

Small mammals, due to their abundance, broad distribution, limited home ranges, and diverse habitats and diets, are frequently in close contact with environmental pollutants [1,2]. Hence, small terrestrial mammals are the best biological monitors for heavy metal exposure in environmental contamination studies [3,4].

The white-footed mouse (Peromyscus leucopus) is particularly valuable in environmental, physiological, and ecotoxicological research for predicting adverse effects on health [5,6]. Previous studies in the Tri-State Mining District (TSMD) have documented the risks associated with toxic metal exposure, noting an increase in blood lead levels and associated mortality rates within the community [7].

Lead (Pb) and Cadmium (Cd) are two metals that are not essential to human biology and have the potential to accumulate in the kidneys, interact with one another, and exacerbate nephrotoxicity [8-10]. Cd, a significant industrial chemical and environmental contaminant, has been linked to an increased risk of kidney disease [11]. Conversely, Pb ions accumulate primarily in kidneys and affect the function of other necessary metals such as zinc and iron [12]. When Pb and Cd accumulate in organs in vivo, they may induce detoxification processes.

According to Sonne, Aspholm, Cd has high binding capacity with the metallothionein (MT-1) protein, a cysteine-rich, with low molecular weight protein that has several essential functions [13,14]. MT-1 has seven metal binding sites, with the α-domain region binding to four atoms and the β-domain binding to three atoms [15]. MT-1s are widely distributed in several taxa and mainly localize in cell cytoplasm and organelles, such as the nucleus and lysosomes of kidney cells [14,16].

MT-1 proteins play critical roles in humans and animals, including heavy metal detoxification and protection against oxidative stress. MT-1 has the ability to bind many metals, including lead, cadmium, zinc, through a chronological, not a cooperative mechanism [17,18]. The MT-1 levels in Algerian mice (Mus spretus) were investigated as a biomarker for elemental toxicity, highlighting the intricate relationship between Zn, MT-1, and cancer, as MT-1 is crucial for regulating intracellular Zn distribution and concentration [19,20].

Our previous work determined the concentrations of these metals in soil samples from the same sites (Tar Creek Superfund Site (TCSFS), Beaver Creek (BC), and two reference sites (Oologah Wildlife Management Area (OWMA) and Sequoyah National Wildlife Refuge (SNWR) (Figure 1) and compared their corresponding levels in kidney of P. leucopus. Additionally, investigated the impact of these metals on bone density and fragility in P. leucopus [21]. The current study aims to elucidate further the relationship between the heavy metal in kidneys and its effect on the induction of MT-1 in P. leucopus. This study focuses on cadmium (Cd), lead (Pb), and zinc (Zn) due to their high concentrations and significant health impacts, prevalence at the Tar Creek Superfund Site (TCSFS), and potential toxicological interactions. This focus allows for a clearer assessment of the most critical contaminants at TCSFS.

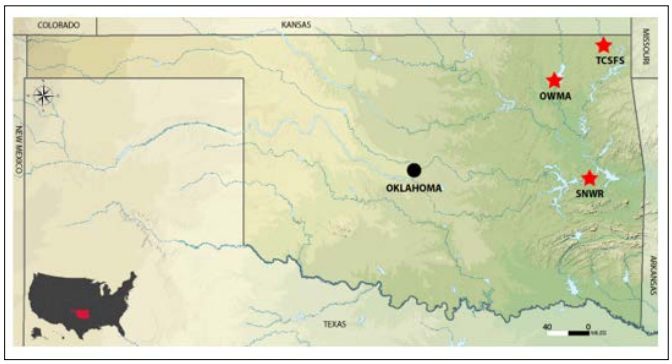

Figure 1: Location of the Tar Creek Superfund Site and two reference study areas (red stars) in northeastern Oklahoma State, USA. OWLA, Oolaga wildlife management area. SNWR, Sequoyah National Wildlife Refuge. TCSFS, Tar Creek Superfund Site

Materials and Methods

Kidney Sampling

Frozen kidney of Peromyscus leucopus were provided from the OSU Collection of Vertebrates, Department of Integrative Biology. Based on the OSU Collection of Vertebrates data, the collecting of Peromyscus leucopus were carried out using standard mark-recapture techniques at the contaminated Tar Creek Superfund Site (Beaver Creek; TCSFS) and two uncontaminated reference sites in Oklahoma, USA: Sequoyah National Wildlife Refuge (SNWR) and Oologah Wildlife Management Area. The data collected by OSU Collection of Vertebrates, recording detailed data on captured mice, including trap location, species, age, sex, reproductive status, body mass, dental condition, parasites, and overall health. Captured mice were euthanized using CO_2 asphyxiation, following approved ethical guidelines. Kidneys were immediately removed, weighed, and stored at -80°C in a liquid nitrogen tank until analysis by the current study.

Tissue preparation and digestion

The preparation and digestion of the kidney subsamples following the method described by Atobatele and Olutona [22]. Each frozen kidney was subsampled into two parts: one part, approximately one-tenth of the whole tissue, was cut in a plastic petri dish on wet ice using a stainless-steel scalpel. These samples were stored in plastic microtubes for MT-1 analysis and returned to the freezer at -80°C without thawing. The second part was stored in plastic microtubes and refrigerated at -40°C before digestion for metal analysis. The kidney samples were then weighed using a digital balance and placed in the tubes of a microwave digestion system (Milestone, Inc, Shelton, Connecticut). For digestion, 1 ml of concentrated nitric acid (99.999% HNO_3) and 0.15 ml of hydrogen peroxide (H_2O_2) were added. The digestion process followed USEPA Method 5031A, taking 50 minutes at 100°C (See Supplementary Information for soil digestion). After digestion, the vessels were carefully depressurized and opened. The samples were then transferred to labeled microtubes for metal analysis (See Supplementary Information for extended methodology).

Heavy Metals Analysis

The concentrations of metals (Pb, Zn, and Cd) in the digested kidney samples were measured using Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS; Perkin Elmer, Shelton, CT, USA) in the OSU Nutrition Science laboratory, according to USEPA Method 6010 [23]. The ICP-MS was calibrated to ensure stability and consistency of results (See Supplementary Information for Quality Assurance and Quality Control (QA/QC) details). Five standard dilutions were prepared before running samples. Each sample, consisting of 80 µl, was mixed with 10 µl of internal standard solution (terbium) and diluted with DDW to a final volume of 5 ml. Sample tubes were vortexed for 20 seconds before analysis.

Metallothionein 1 Analysis

The kidney subsamples, of part one, were homogenized following the Invitrogen MT protocols (Uscn, Life Science Inc., Houston, TX). Each subsample was pulverized in liquid nitrogen and transferred into a pre-chilled 5 ml culture tube. 10 µl of protease inhibitor (Cat. No 78441B Sigma Aldrich, St. Louis, MO) was mixed with 1 ml phosphate buffered saline (PBS) and cooled using wet ice. Tissues were homogenized at low speed for ~20 seconds, then transferred into microcentrifuge tubes and centrifuged at 14,000 x g at 4°C for 15 min. The supernatants were removed, and two 100 µl aliquots were stored at -80°C for MT-1 analysis by ELISA (Uscn Life Science Inc, Huston, TX).

Next, 100 µl of sample or standard was added to the appropriate wells in the microtiter plate. 50 µl of conjugate was then added to each well and mixed thoroughly. The microplate was covered and incubated for one hour at 37°C in a humid chamber. After incubation, each well was washed five times with 300-400 µl 1X Wash solution, and after the last wash, the plate was inverted and blotted dry by tapping on absorbent paper. Next, 50 µl each of Substrate A and Substrate B were added to each well. The plate was then covered and incubated for 10-15 minutes at room temperature, protected from light. After incubation, 50 µl of Stop solution was added to each well and mixed well. The optical density (O.D.) was read immediately at 450 nm. For data calculation, the mean blank value was subtracted from each sample or standard value, and the mean for duplicate wells was calculated. A standard curve was constructed using Gen5 Microplate Reader and Imager Software (catalog no. GEN5, BioTek), providing the quantitative measurement of MT1 concentration in each sample as visualized by the colorimetric reaction at 450 nm.

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences (SPSS) software (V 25) and GraphPad Prism (V 10). The homogeneity of variances was assessed using Levene's test, and the Shapiro-Wilk test was employed to check for normal distribution of the data prior to conducting an Analysis of Variance (ANOVA). Post-hoc comparisons of means were performed using the Tukey test. Data are presented as mean ± standard error, with different superscript letters indicating significantly different mean values. Statistically non-significant and significant outputs were accompanied by symbols NS and*, respectively, with the data tested at a significance level of $P \leq 0.05$.

Results and Discussion

Metal Concentrations in the Kidney

Concentrations of heavy metals in the kidneys (Tables 1 and 2) indicate that Zn levels did not differ among polluted Tar Creek Superfund Site (TCSFS) and the reference sites OWMA and SNWR (See Supplementary Data). However, Cd levels (µg/kg) were significantly higher in kidney samples from TCSFS compared to the reference sites ($P < 0.05$). Pb concentrations were also elevated in samples from TCSFS relative to OWMA and SNWR (Tables 1,2). The body weights of P. leucopus were consistent across the study, showing no significant differences between individuals from contaminated and reference sites (Table 2 and Supplementary Information).

Table 1: The Average Concentrations (µg/Kg) of Zn, Pb, and Cd in the Soil and Kidney Samples from P. Leucopus Collected from the Studied Sites

Site	Metal	Soil Conc.	Kidney Conc.
Tar Creek Superfund Site (TCSFS)	Zn	14,083.9 ± 1825.8	23.1 ± 3.3
	Pb	1,132 ± 278	0.57 ± 0.10
	Cd	48.04 ± 3.98	4.62 ± 0.71
Oologah Wildlife Management Area (OWMA)	Zn	52.6 ± 5.0	18.5 ± 3.8
	Pb	6.4 ± 1.1	0.04 ± 0.01
	Cd	0.15 ± 0.03	0.53 ± 0.08
Sequoyah National Wildlife Refuge (SNWR)	Zn	20.0 ± 1.9	28.4 ± 4.6
	Pb	2.3 ± 0.3	0.50 ± 0.01
	Cd	0.06 ± 0.01	0.53 ± 0.06

Table 2: Body weight (g), heavy metals concentrations in the kidney (µg/Kg), and Metallothionein concentrations (µg/mg protein) from P. leucopus collected from the studied sites. Values with different superscript letters (a, b) in the same column for each parameter are significantly different (P ≤ 0.05)

			Heavy metal conc. in the kidney			
Site	**n**	**BW**	**Pb**	**Cd**	**Zn**	**MT-1**
TCSFS	17	25±0.8a	0.57±0.10a	4.62±0.71a	23.1±3.3a	0.15±0.07a
OWMA	16	24±1.3a	0.04±0.01b	0.53±0.08b	18.5±3.8a	0.08±0.02a
SNWR	16	23±1.2a	0.5±0.01b	0.53±0.06b	28.4±4.6a	0.22±0.06a

Supplementary Information

Part 1
Extended Methodology
Sample Collection
Kidneys of Peromyscus Leucopus
Frozen kidney of Peromyscus leucopus were provided from the OSU Collection of Vertebrates, Department of Integrative Biology (See the Main text).

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

Sample Preparation
Kidney Samples Preparation and Digestion
Each kidney was subsampled by dividing it into two parts: one part was cut in a plastic petri dish placed on wet ice using a stainless-steel scalpel to approximately one-tenth of the whole tissue. These samples were saved in plastic microtubes for metallothionein-1 analysis and returned to the freezer at -80°C without thawing. The second part was saved in plastic microtubes and refrigerated at -40°C prior to digestion for metal analysis.

The kidney samples were then weighed using a digital balance and small microwave tubes were used for kidney sample digestion. Each sample was placed in the microwave tubes of the digestion system (Milestone, Inc, Shelton, Connecticut). For the digestion process, 1 ml of concentrated (99.999%) nitric acid (HNO3) was added to the tube containing the kidney sample, followed by 0.15 ml of and hydrogen peroxide (H_2O_2). This digestion process was conducted using a microwave digestion system (Milestone, Inc, Shelton, Connecticut), following the USEPA Method Number 5031A. The microwave operation took 50 minutes at a temperature of 100°C to complete the digestion. Next, the vessels were pulled gently from the microwave after 5 minutes. The segments were opened slowly using a torque wrench to release the pressure from the vessels. Samples were transferred to the hood to open the vessels cover, and yellow acid evaporated from the vessels. After the digestion, 1 ml of the acid solution was transferred into labeled microtubes for metal analysis.

For metallothionein-1 analysis, kidney subsamples, of part one, were homogenized using Invitrogen MT protocols. Each sample was crushed in liquid nitrogen and transferred into a pre-chilled 5 ml culture tube. Ten µl of protease inhibitor was mixed with 1 ml of phosphate-buffered saline (PBS) and cooled using wet ice. The tissues were homogenized at low speed for about 20 seconds, then transferred into micro centrifuge tubes and centrifuged at 14,000 x g at 4°C for 15 minutes. The supernatants were removed, and four 80 µl aliquots were stored at -80°C for total protein and metallothionein-1 analysis using ELISA procedures.

Soil Samples Preparation and Digestion
Soil samples were collected from various sites, including the Tar Creek Superfund Site (TCSFS) and reference sites such as Sequoyah National Wildlife Refuge (SNWR) and Oologah Wildlife Management Area (OWMA). The samples were dried at room temperature for two weeks, then sieved through a 1 mm

sieve followed by a 250 µm sieve. The sieved soil was stored in labeled acid-washed glass bottles.

Soil Digestion

Soil samples were digested in the Nutrition Science Department's laboratory using microwave digestion system (Milestone, Inc, Shelton, Connecticut) specified by USEPA Method Number 3051A (Kingston et al., 1997; United States Environmental Protection Agency, 1986) according to the EPA procedure. A standard soil sample from a reference site was randomly selected and marked as a control sample. All soil samples from the TCSFS and the two reference sites, SNWR and OWMA, were weighed using a digital balance (AIDINGER COMP) and labeled polypropylene plates. Soil samples were digested according to the EPA procedure using microwave digestion, with each sample size being 0.5 g. The standard soil sample was placed in vessel No.1 for probe detection and drawing a standard curve. A double distilled trace element grade nitric acid (99.999%) was added to the vessels, and the mixture was digested in a microwave at 100°C for 50 minutes (following the US EPA method 5031 A).

After digestion, the vessels were allowed to cool, and the digested solution was transferred to plastic tubes. The vessels were then rinsed with double distilled water, and the rinsate was combined with the digested solution. The samples were centrifuged at 1,200 g for 10 minutes to separate the solid residue from the liquid, with the supernatant transferred to new tubes for metal analysis. The samples were then diluted with double distilled water (DDW) and prepared for Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) analysis. The samples were decanted into a new tube (2nd tube) gently and slowly to avoid solution contamination. The first tube that contained the soil was disposed of separately. The second tube for each digested sample was labeled as stock that was used for metal analysis. A total of 0.2 ml of the digested soil solution in 10 ml total volume samples were diluted by adding double distilled water (DDW) to 0.20 ml sample solution in the new tube (3rd tube). This tube of each sample was used for the Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS) instrument (Perkin Elmer) to analyze metal (Pb, Zn, and Cd) concentrations in each sample separately.

Metal Measurement

The concentrations of metals (Pb, Zn, and Cd) in both the digested soil and kidney samples were measured using Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS; Perkin Elmer, Shelton, CT, USA) in the OSU Nutrition Science laboratory, according to USEPA Method 6010 (United States Environmental Protection Agency, 1996). The ICP-MS was calibrated to ensure stability and consistency of results. Calibration involved preparing five standard dilutions and using terbium as an internal standard. For soil samples, 0.1 µl was diluted to 10 ml with double distilled water (DDW), with 20 µl of the internal standard added. Sample tubes were vortexed for 20 seconds before analysis. For kidney subsamples, the ICP-MS instrument was also calibrated using the same method (See the Main text).

ELISA Test Procedures

The kidney samples were homogenized following the Invitrogen MT protocols (Uscn, Life Science Inc., Houston, TX). For sample preparation, frozen kidney samples (1 mg) were crushed in liquid nitrogen and transferred into pre-chilled 5 ml culture tubes. 10 µl of protease inhibitors (Cat. No 78441B Sigma Aldrich, St. Louis, MO) was mixed with 1 ml Phosphate Buffered Saline (PBS) and cooled using wet ice. The tissues were then homogenized at low speed for about 20 seconds, with the samples kept cool in wet ice. Subsequently, the samples were transferred into 1.7 ml microcentrifuge tubes and centrifuged at 14,000 x g at 4°C for 15 minutes. The supernatant was removed and divided into two 100 µl aliquots for MT-1 analysis by ELISA (Uscn Life Science Inc, Huston, TX).

Next, 100 µl of sample or standard was added to the appropriate wells in the supplied microtiter plate. 50 µl of conjugate was then added to each well and mixed thoroughly. The microplate was covered and incubated for one hour at 37°C in a humid chamber. After incubation, each well was washed five times with 300-400 µl 1X Wash solution per well, and after the last wash, the plate was inverted and blotted dry by tapping on absorbent paper. Next, 50 µl of Substrate A was added to each well, followed by the addition of 50 µl of Substrate B. The plate was then covered and incubated for 10-15 minutes at room temperature. Because the substrate is light-sensitive, it was kept out of direct sunlight or covered with foil. Following the incubation period, 50 µl of Stop solution was added to each well and mixed well. The optical density (O.D) was read immediately at 450 nm. For data calculation, the mean blank value was subtracted from each sample or standard value to calculate the mean for duplicate (or greater) wells. A standard curve was constructed using Gen5 Microplate Reader and Imager Software (catalog no. GEN5, BioTek), which provided the quantitative measurement of MT1 concentration in each sample as visualized by the colorimetric reaction at 450 nm.

Quality Assurance and Quality Control (QA/QC)

Instrument Calibration

The ICP-MS machine was calibrated according to USEPA method 6010 (USEPA, 1996) to ensure stability and consistency of results. Five standard dilutions were prepared using internal standard solution and DDW. All samples were vortexed before running in the ICP-MS. Metal concentrations (Pb, Cd, and Zn) were determined, and results that were not within the standard range were reanalyzed.

Limits of Detection (LODs)

The LODs for metals were determined based on three times the standard deviation of the blank using three-second integration time and peak hopping at 1-point per mass: Cadmium (Cd): 0.4 ng/L, Zinc (Zn): 0.4 ng/L, and Lead (Pb): 9 ng/L.

Certified Reference Materials (CRMs)

CRMs from NIST, such as Standard Reference Material 1643f for trace elements in water, were used for instrument calibration and method validation. CRMs were analyzed at regular intervals (every 10 samples) to ensure accuracy.

Calibration and Instrument Maintenance

Instruments were calibrated daily, and internal standards were used to monitor performance. Detailed records of all calibration procedures were maintained.

Sample Handling and Preparation

To prevent contamination, field, trip, and laboratory blanks were used. Soil and kidney samples were handled with care, ensuring that all equipment was properly cleaned and that samples were stored appropriately.

Replicates and Duplicates

We analyzed replicate samples to ensure precision and handled duplicate samples consistently.

Spike Recovery: Spike recovery experiments demonstrated recovery rates of 95% for lead, 94% for cadmium, and 97% for zinc, indicating high method accuracy.

Control Samples
Control samples from unexposed Peromyscus leucopus provided baseline data for comparison.

Data Validation
Statistical methods were employed to validate the data, and quality control charts were used to monitor performance over time.

Part 2
Extended Results
Extended ELISA Test Results
The blanks exhibited a low average absorbance of 0.126 with a standard deviation of 0.013. This minimal variability indicates that background noise is not significantly affecting the measurements, ensuring that the absorbance values obtained for the standards reflect specific binding rather than noise.

For the standards, the variability in absorbance values varied across different concentrations. At high concentrations, such as STD1 (1000 ng/mL) and STD2 (500 ng/mL), the standard deviations were very low, at 0.013 and 0.011 respectively. This low variability suggests that the assay provides accurate and consistent results at these levels. As the concentration decreases, the standard deviations increase. For instance, STD3 (250 ng/mL) showed a standard deviation of 0.026, and STD4 (125 ng/mL) had a standard deviation of 0.020. These moderate variabilities are expected and indicate that the assay maintains reasonable precision at mid-range concentrations. At lower concentrations, the standard deviations are notably higher, reflecting the challenges of detecting lower signal levels. For STD5 (62.5 ng/mL), the standard deviation was 0.050, and it increased further to 0.074 for STD6 (31.3 ng/mL), 0.076 for STD7 (15.6 ng/mL), and 0.077 for STD8 (7.8 ng/mL). This increased variability at lower concentrations highlights the decreased precision and accuracy typical of ELISA assays at low signal levels due to a higher signalto-noise ratio. The standard deviations for higher concentration standards (e.g., STD1 and STD2) are lower compared to lower concentration standards (e.g., STD5 and STD6). This suggests that higher concentrations provide more consistent results, while lower concentrations show increased variability. This increased variability at lower concentrations is typical in assays due to the lower signal-to-noise ratio.

In summary, the analysis confirms that the assay performs reliably at high concentrations with low error, while variability increases at lower concentrations, reflecting common challenges in ELISA testing. We hope this detailed explanation addresses your concerns effectively.

Sample No.	Mouse OK no.	Site	Zn. Conc.	Cd.Conc.	Pb.Conc.	MT
18	6251	TCSFS	2578.156	294.333	78.522	29466.5
18'	6251	TCSFS	2648.872	292.198	78.849	
6	6252	TCSFS	1846.627	87.677	51.971	1416325
6'	6252	TCSFS	1805.708	88.016	51.229	
50	6253	SNWR	2318.058	101.535	3.194	368089.8
50'	6253	SNWR	2274.236	101.087	3.015	
10	6254	TCSFS	1221.618	1018.922	28.515	1011523
10'	6254	TCSFS	1202.834	1024.475	27.51	
1	6255	TCSFS	3091.301	523.054	25.071	104340.4
1'	6255	TCSFS	3020.702	528.471	24.497	
8	6256	TCSFS	1641.956	1654.939	283.78	337110.6
8'	6256	TCSFS	1586.687	1598.518	279.234	
4	6257	TCSFS	921.925	150.467	25.321	198325.8
4'	6257	TCSFS	925.576	153.663	24.964	
3	6258	TCSFS	1703.024	86.511	51.706	.
3'	6258	TCSFS	1660.054	85.367	50.972	
15	6258	TCSFS	2252.37	90.935	54.915	93230.1
15'	6258	TCSFS	2253.696	90.165	54.646	
2	6259	TCSFS	2827.013	42.292	89.335	151680.1
2'	6259	TCSFS	2833.758	44.302	88.436	
12	6260	TCSFS	569.663	15.348	14.403	322440.9
12'	6260	TCSFS	559.214	15.127	14.057	
14	6261	TCSFS	1023.076	475.475	40.984	.
14'	6261	TCSFS	980.381	468.007	40.009	
9	6264	TCSFS	8274.281	336.567	53.624	245370.8
9'	6264	TCSFS	8292.728	337.814	54.005	
7	6265	TCSFS	1046.774	23.628	49.779	97336.1
7'	6265	TCSFS	1044.739	24.587	49.523	

13	6266	TCSFS	8710.13	433.892	41.068	156993.8
13'	6266	TCSFS	8768.515	443.56	41.527	
17	6267	TCSFS	1095.472	730.809	37.274	194672.2
17'	6267	TCSFS	1054.848	713.748	36.511	
5	6268	TCSFS	1597.823	1251.866	19.516	399971.6
5'	6268	TCSFS	1610.762	1288.379	19.42	
11	6269	TCSFS	1060.957	704.835	36.399	58450
11'	6269	TCSFS	1037.226	705.387	35.653	
48	6271	SNWR	1093.866	53.524	3.823	148729.3
48'	6271	SNWR	1093.342	53.073	3.573	
44	6272	SNWR	4145.941	135.183	4.315	1383477
44'	6272	SNWR	4091.168	133.417	4.311	
49	6276	SNWR	3815.254	124.672	4.236	556482.4
49'	6276	SNWR	3791.705	123.58	3.72	
43	6278	SNWR	9390.973	47.759	11.194	152120.5
43'	6278	SNWR	9388.505	46.979	11.364	
40	6279	SNWR	2152.396	17.568	9.182	104340.4
40'	6279	SNWR	2124.484	16.963	9.288	
36	6280	SNWR	1167.214	5.811	5.893	926504.4
36'	6280	SNWR	1264.551	6.455	6.51	
38	6281	SNWR	942.872	45.564	2.977	181629.6
38'	6281	SNWR	953.78	46.034	2.927	
42	6282	SNWR	6771.614	47.954	4.837	288868.4
42'	6282	SNWR	6700.898	46.994	4.83	
34	6283	SNWR	716.827	45.45	2.373	711999.6
34'	6283	SNWR	683.271	43.051	2.539	
46	6284	SNWR	1702.667	55.862	2.372	.
46'	6284	SNWR	1718.428	56.652	2.438	
16	6299	TCSFS	1623.166	984.65	27.27	111378.2
16'	6299	TCSFS	1622.437	983.188	27.247	
37	6301	SNWR	1613.943	17.287	9.271	196121.4
37'	6301	SNWR	1589.222	17.133	9.165	
35	6302	SNWR	1673.368	34.239	5.838	173900.8
35'	6302	SNWR	1671.686	34.236	5.926	
41	6307	SNWR	1077.599	32.162	3.236	623868.9
41'	6307	SNWR	1054.943	31.535	3.161	
45	6308	SNWR	1544.026	32.53	3.973	561554.4
45'	6308	SNWR	1543.301	32.587	4.031	
47	6309	SNWR	1509.86	5.273	1.668	202884.2
47'	6309	SNWR	1483.146	4.478	1.159	
24	6311	OWMA	1387.405	84.88	1.926	7856.7
24'	6311	OWMA	1332.932	82.806	1.811	
29	6312	OWMA	779.437	31.426	1.363	.
29'	6312	OWMA	750.264	31.176	1.154	
22	6313	OWMA	9652.038	145.435	26.606	1012489
22'	6313	OWMA	9682.633	145.256	26.364	
31	6315	OWMA	912.57	12.938	1.136	234774.6
31'	6315	OWMA	891.422	12.754	1.052	

23	6316	OWMA	3186.105	177.273	3.944	200710.5
23'	6316	OWMA	3081.882	174.042	3.912	
21	6317	OWMA	1278.832	40.292	4.752	48164.06
21'	6317	OWMA	1227.178	39.389	4.611	
28	6318	OWMA	627.593	76.271	1.434	54560.34
28'	6318	OWMA	607.938	74.703	1.376	
33	6321	OWMA	1608.66	34.263	4.546	72458.6
33'	6321	OWMA	1588.907	34.008	4.479	
20	6322	OWMA	1497.239	34.443	4.807	318257.8
20'	6322	OWMA	1457.989	33.102	4.551	
30	6323	OWMA	739.202	49.057	1.518	234819.7
30'	6323	OWMA	653.246	49.032	0.98	
19	6325	OWMA	1305.984	31.562	1.339	33089.4
19'	6325	OWMA	1260.479	31.388	1.255	
26	6326	OWMA	658.731	18.068	0.371	919741.6
26'	6326	OWMA	635.603	17.881	0.318	
25	6444	OWMA	645.102	9.915	8.303	43492.13
25'	6444	OWMA	620.3334	10.026	8.253	
32	6445	OWMA	680.232	45.816	0.641	270498
32'	6445	OWMA	659.81	45.2	0.508	
27	6448	OWMA	7286.199	94.67	4.021	186460.2
27'	6448	OWMA	7240.486	93.233	3.59	

The distribution of heavy metals in the animal environment and their accumulation in animal tissues have been significantly correlated in previous studies [24-27]. On the other hand, animals exposed to heavy metals experienced diverse tissue effects [21,28-32]. This study is among the first to document MT-1 induction in P. leucopus, providing a foundation for future research on the physiological responses of small mammals to contaminated habitats in North America. Notably, our study has demonstrated that the kidneys of P. leucopus accumulate Pb, Cd, and Zn, three heavy metals. These findings indicate and predict that these hazardous elements may accumulate in other vital organs, such as the liver, intestines, and spleen. Accordingly, some endpoints like tooth abnormalities, genetic structure, and anogenital distance have already been examined in P. leucopus, and another study investigated population dynamics and demographics at the same locations [33,34]. The findings of the heavy metal concentration in this study agreed with those of previous studies. The current study agrees with Levengood and Heske, who found that compared to nearby reference sites, P. leucopus accumulated higher burdens from heavy metals- contaminated areas [35]. Similarly, the abundance of heavy metals in soil and their concentrations in the liver and kidney of various small mammal species are significantly correlated [36].

Metallothionein 1 Concentrations in the Kidney

MT-1 concentrations exhibited high variability among P. leucopus specimens from TCSFS, and the reference sites, OWMA and SNWR, as shown in Table 1. Additionally, the correlation between MT-1 concentrations and heavy metal levels (Zn, Cd, Pb) in P. leucopus kidneys at three study sites revealed varying relationships (Tables 3-5). At TCSFS, correlations between MT-1 and Zn, Cd, and Pb were -0.1494, 0.0736, and -0.0194, respectively, indicating generally weak relationships with no significance. The OWMA site showed moderate positive correlations between MT-1 and Zn ($r = 0.4911$), MT-1 and Cd ($r = 0.2165$), and MT-1 and Pb ($r = 0.5533$), with the correlations between MT-1 and Zn, and MT-1 and Pb being significant ($P < 0.05$). SNWR demonstrated a weak negative correlation between MT-1 and Zn ($r = -0.0655$), a moderate positive correlation between MT-1 and Cd ($r = 0.4524$), and a weak negative correlation between MT-1 and Pb ($r = -0.2724$), with the correlation between MT-1 and Cd being significant ($P < 0.01$). Overall, the results suggest that the relationship between MT-1 concentrations and heavy metals vary across different sites (See Supplementary Information for extended results).

Metallothionein plays a pivotal role in the homeostasis of essential metals such as zinc and in detoxifying non-essential toxic metals [16]. The mechanisms by which MTs confer protection against heavy metal toxicity involve their high cysteine content and capacity for metal binding, which neutralizes the reactive properties of heavy metals and prevents cellular damage. Despite the established role of MTs in detoxification, our study found no significant differences in MT-1 concentrations in the kidneys of P. leucopus from contaminated and reference sites. This unexpected result raises questions about the adaptive mechanisms employed by this species and suggests that MT-1 levels may not solely reflect the heavy metal burden. Factors such as the bioavailability of metals, the duration of exposure, and individual physiological differences, including age, weight, and sex, may influence MT-1 expression.

Table 3: Correlation between Metallothionein-1 conc. (µg/mg) and heavy metal levels (µg/Kg) in TCSFS. CI: Confidence Interval; ns: not significant

Pearson r	MT vs. Zn	MT vs. Cd	MT vs. Pb
r	-0.1494	0.07358	-0.01940
95% CI	-0.4734 to 0.2102	-0.2824 to 0.4117	-0.3656 to 0.3315
P (two-tailed)	0.41 (ns)	0.69 (ns)	0.92 (ns)
Number of XY Pairs	32	32	32

Table 4: Correlation between Metallothionein-1 conc. (µg/mg) and heavy metal levels (µg/Kg) in OWMA. CI: Confidence Interval; ns: not significant; *: significant

Pearson r	MT vs. Zn	MT vs. Cd	MT vs. Pb
r	0.4911	0.2165	0.5533
95% CI	0.1445 to 0.7304	-0.1703 to 0.5455	0.2271 to 0.7679
P (two-tailed)	0.01*	0.27 (ns)	0.01*
Number of XY Pairs	28	28	28

Table 5: Correlation between Metallothionein-1 conc. (µg/mg) and heavy metal levels (µg/Kg) in SNWR. CI: Confidence Interval; ns: not significant; *: significant

Pearson r	MT vs. Zn	MT vs. Cd	MT vs. Pb
r	-0.06548	0.4524	-0.2724
95% CI	-0.4159 to 0.3019	0.1101 to 0.6988	-0.5762 to 0.09738
P (two-tailed)	0.73 (ns)	0.01*	0.15 (ns)
Number of XY Pairs	30	30	30

Furthermore, the correlation results suggest that despite elevated levels of heavy metals at the TCSFS, MT-1 concentrations, and heavy metal levels showed no significant relationship. This could be due to various factors. First, while heavy metals are present in high concentrations, the specific bioavailability and uptake by the kidneys might vary, leading to inconsistent MT responses. Second, MT-1 expression could be influenced by other stressors or biological factors not directly related to metal concentration, such as animal age, diet, or other environmental contaminants. While our current study did not include sex and age as covariates due to limitations in sample size and age precision, we recognize the importance of these factors. Future research should aim to address these limitations by incorporating larger sample sizes, precise age data, and multivariate analyses. This approach will enhance our understanding of the physiological responses to heavy metal exposure in Peromyscus leucopus and provide more robust insights into the effects of these variables.

Finally, the weak correlations at this site could indicate a threshold effect, where only extremely high metal levels trigger a substantial MT response. In contrast, the OWMA site showed moderate positive correlations between MT-1 and Zn and Pb, suggesting that these metals might play a more significant role in inducing MT-1. The results from the SNWR site indicated a moderate positive correlation between MT-1 and Cd, which could be related to varying bioavailability and uptake of these metals by the kidney.

These findings suggest that MT alone may not be a reliable indicator of heavy metal contamination levels. Additionally, our study underscores the complexity of the biological response to heavy metals and highlights the need for further studies to understand the factors influencing MT-1 expression in small mammals.

Ethical Approval

The Oklahoma State University Institutional Animal Care and Use Committee approved the experimental procedures used in this study (approval no. AS056).

Competing Interests

The authors declare that they have no conflict of interest.

References

1. Gaukler S, Murphy S, Berryhill J, Brent E Thompson, Benjamin J Sutter, et al. (2020) Investigating effects of soil chemicals on density of small mammal bioindicators using spatial capture-recapture models. PloS one 15: e0238870.
2. Balčiauskas L, Ežerinskis Ž, Stirkė V, Laima Balčiauskienė, Andrius Garbaras, et al. (2022) The elemental composition of small mammals in a commercial orchard–meadow system. Chemosphere 296: 134048.
3. Koroleva E, Miholova D, Cibulka J, Mader P, Slameva A (1999) Small Mammals as Bioindicators for Terrestrial Ecosystems in Bohemia. Biomarkers: A Pragmatic Basis for Remediation of Severe Pollution in Eastern Europe 308-309.
4. Wijnhoven S, Leuven RSEW, Van Der Velde G, Jungheim G, Koelemij E I, et al. (2007) Heavy-Metal Concentrations in Small Mammals from a Diffusely Polluted Floodplain: Importance of Species- and Location-Specific Characteristics. Archives of Environmental Contamination and Toxicology 52: 603-613.
5. Mineau P (2006) Ecotoxicology of Wild Mammals. Edited by Richard F. Shore Barnett A. Rattner. John Wiley and Sons Ltd., Chichester, United Kingdom. Wildlife Society Bulletin 34: 250-251.
6. Husby MP, Hausbeck JS, McBee K (1999) Chromosomal aberrancy in white-footed mice (Peromyscus leucopus) collected on abandoned coal strip mines, Oklahoma, USA [print]. Environmental Toxicology and Chemistry 18: 919-925.
7. Park H, Noh K, Min JJ, Christopher Rupar (2020) Effects of Toxic Metal Contamination in the Tri-State Mining District on

the Ecological Community and Human Health: A Systematic Review. International Journal of Environmental Research and Public Health 17: 6783.
8. Shefa ST, Héroux P (2017) Both physiology and epidemiology support zero tolerable blood lead levels. Toxicology Letters 280: 232-237.
9. Daley GM, Pretorius CJ, Ungerer JP (2018) Lead toxicity: an Australian perspective. The Clinical Biochemist Reviews 39: 61.
10. Satarug S, Gobe CG, Vesey AD, Kenneth R Phelps (2020) Cadmium and lead exposure, nephrotoxicity, and mortality. Toxics 8: 86.
11. Prozialeck WC, Edwards JR (2012) Mechanisms of cadmium-induced proximal tubule injury: new insights with implications for biomonitoring and therapeutic interventions. Journal of Pharmacology and Experimental Therapeutics 343: 02-12.
12. Damek Poprawa M, Sawicka Kapusta K (2003) Damage to the liver, kidney, and testis with reference to burden of heavy metals in yellow-necked mice from areas around steelworks and zinc smelters in Poland. Toxicology 186: 01-10.
13. Sonne C, Aspholm O, Dietz R, Steen Andersen, Marc H G Berntssen, et al. (2009) A study of metal concentrations and metallothionein binding capacity in liver, kidney and brain tissues of three Arctic seal species. Science of the total environment 407: 6166-6172.
14. Sigel A, Sigel H, Sigel RK (2015) Metallothioneins and related chelators Vol 5. Walter de Gruyter GmbH & Co KG.
15. Krezel A, Maret W (2021) The bioinorganic chemistry of mammalian metallothioneins. Chemical reviews 121: 14594-14648.
16. Ziller A, Fraissinet Tachet L (2018) Metallothionein diversity and distribution in the tree of life: a multifunctional protein. Metallomics 10: 1549-1559.
17. Dallinger R (2024) Metals and metallothionein evolution in snails: a contribution to the concept of metal-specific functionality from an animal model group. BioMetals 37: 671-696.
18. Davis SR, Cousins RJ (2000) Metallothionein Expression in Animals: A Physiological Perspective on Function. The Journal of Nutrition 130: 1085-1088.
19. Marques CC, Gabriel SI, Pinheiro T, Ana Maria Viegas Crespo, Maria da Luz Mathias, et al. (2008) Metallothionein levels in Algerian mice (Mus spretus) exposed to elemental pollution: An ecophysiological approach. Chemosphere 71: 1340-1347.
20. Ruttkay Nedecky B, Nejdl L, Gumulec J, Ondrej Zitka, Michal Masarik, et al. (2013) The Role of Metallothionein in Oxidative Stress. International journal of molecular sciences 14: 6044-6066.
21. Elturki M (2022) 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. https://peerj.com/articles/14605/.
22. Atobatele OE, Olutona GO (2015) Distribution of three non-essential trace metals (Cadmium, Mercury and Lead) in the organs of fish from Aiba Reservoir, Iwo, Nigeria. Toxicology reports 2: 896-903.
23. (1996) Environmental Protection Agency U. Method 6010B: Inductively coupled plasma-atomic emission spectrometry. SW-846 test methods for evaluating solid wastes.
24. Said R, Rab AGG, Khattab NM, Samy A Saber, Boshra A El Salkh (2023) Hepatic Alterations in the Amphibian Sclerophrys regularis as Biomarkers for Habitat Destruction. Journal of Chemical Health Risks 2: 285-290.
25. Said REM, Ashry M, AbdAllah EM (2021) The use of biomarkers in the Nile Tilapia (Oreochromis niloticus) as biological signals to track Nile contamination in Egypt. Egyptian Journal of Aquatic Biology and Fisheries 25: 203-214.
26. Abdel Wahab M, Yassien MH, Thabet AA, Rashad Said, Aldoushy Mahdy, et al. (2022) Seasonal variations of some heavy metal concentrations in seawater, sediment, and the surf clam, Mactra olorina (Philippi, 1846) in the Great Bitter Lake, Suez Canal, Egypt. Egyptian Journal of Aquatic Biology and Fisheries 26: 83-98.
27. Khattab NMA, Saber SA, El Salkh BA, Rashad E. M. Said (2021) Genotoxicity and limbs asymmetry in the Egyptian toad (Sclerophrys regularis) as biomarkers for heavy metals toxicity. Egyptian Journal of Aquatic Biology and Fisheries 25: 705-717.
28. Farrag MMS, Said RE, Elmileegy IMH, Khalil Nasser S Abou, Allah Eman SA, et al. (2022) Effects of penconazole and copper nanoparticle fungicides on redbelly tilapia, Coptodon zillii (Gervais, 1848): Reproductive outcomes. International Journal of Aquatic Biology 9: 370-382.
29. Lavery TJ, Kemper CM, Sanderson K, Christopher G Schultz, Peter Coyle, et al. (2009) Heavy metal toxicity of kidney and bone tissues in South Australian adult bottlenose dolphins (Tursiops aduncus). Marine Environmental Research 67: 1-7.
30. Osman AGM, Mekkawy IA, Verreth J, Frank Kirschbaum (2007) Effects of lead nitrate on the activity of metabolic enzymes during early developmental stages of the African catfish, Clarias gariepinus (Burchell, 1822). Fish Physiology and Biochemistry 33: 01-13.
31. Hamed M, Soliman HAM, Sayed AE DH (2019) Ameliorative effect of Spirulina platensis against lead nitrate–induced cytotoxicity and genotoxicity in catfish Clarias gariepinus. Environmental Science and Pollution Research 26: 20610-20618.
32. Xie J, Zhou F, Ouyang L, Qi Li, Shaoqi Rao, et al. (2023) Insight into the effect of a heavy metal mixture on neurological damage in rats through combined serum metabolomic and brain proteomic analyses. Science of The Total Environment 895: 165009.
33. Hays KA (2010) An ecological study of white-footed mice (Peromyscus leucopus) from Tar Creek Superfund Site. https://openresearch.okstate.edu/server/api/core/bitstreams/cd79b85f-bc87-431e-98bc-01e325dc2bbf/content.
34. Kendra LP, Karen M (2010) Population Parameters of Peromyscus leucopus (White-Footed Deermice) Inhabiting a Heavy Metal Contaminated Superfund Site. The Southwestern Naturalist 55: 363-373.
35. Levengood JM, Heske EJ (2008) Heavy metal exposure, reproductive activity, and demographic patterns in white-footed mice (Peromyscus leucopus) inhabiting a contaminated floodplain wetland. Science of The Total Environment 389: 320-328.
36. Fritsch C, Cosson RP, Coeurdassier M, Francis Raoul, Patrick Giraudoux, et al. (2010) Responses of wild small mammals to a pollution gradient: Host factors influence metal and metallothionein levels. Environmental Pollution 158: 827-840.