Published in final edited form as:

Sci Total Environ. 2015 June 15; 0: 526–533. doi:10.1016/j.scitotenv.2015.03.013.

Hair and Bone as Predictors of Tissular Mercury Concentration in the Western Alaska Red Fox, *Vulpes vulpes*

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Abstract

We evaluated if total mercury (THg) concentrations of keratin-based and bone-based tissues can predict THg concentrations in skeletal muscle, renal medulla, renal cortex, and liver. The THg concentration in matched tissues of 65 red foxes, *Vulpes vulpes*, from western Alaska was determined. Hair THg concentration had a significant positive correlation with liver, renal medulla, renal cortex, and muscle. The THg concentration for males and females is moderately predictive of THg concentration in the renal cortex and liver for these foxes based on R^2 values ($R^2 = 0.61$ and 0.63 , respectively). Bone is weakly predictive of THg concentration in muscle ($R^2 = 0.36$), but not a reliable tissue to predict THg concentration in liver ($R^2 = 0.28$), renal cortex ($R^2 = 0.33$), or renal medulla ($R^2 = 0.29$). These results confirm the potential use of trapped animals, specifically foxes, as useful Arctic sentinel species to inform researchers about patterns in THg levels over time as industrialization of the Arctic continues.

Keywords

Mercury; Arctic; Bone; Hair; Kidney; Sentinel species

1 Introduction

Globally, mercury (Hg) is a naturally occurring element found throughout the environment in air, water, and soil. It exists in several forms: elemental (metallic mercury), inorganic

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(mercuric chloride) and organic (methylmercury) compounds (Clarkson and Magos 2006). It is released into the atmosphere and water by volcanic activity, weathering of rocks; and, anthropogenic mercury is through industry emissions from agriculture, manufacturing, and mining (Bilandzic et al. 2010; Clarkson 1997; Clarkson and Magos 2006; Kalisinska et al. 2012). In ecotoxicological studies, total mercury (THg) is mainly determined as the sum of organic (mostly as methylmercury) and inorganic mercury (Eisler 1987). Many organisms are exposed through the food web and are sensitive to the effects of mercury, especially monomethylmercury (MeHg^+) (Brookens et al. 2008; Dietz et al. 2009). The degree of food web biomagnification of MeHg^+ depends on dietary patterns, as well as age and sex (Bilandzic et al. 2010; Clarkson and Magos 2006; Cumbie and Jenkins 1974; Knott et al. 2011a; Wren 1986).

With increased release of Hg into the environment and potential methylation, tissue concentrations in foxes will likely increase; therefore, it is important to continue biomonitoring Hg concentrations in terrestrial ecosystems over time in medium-sized carnivore mammals especially ones that are numerous and widely distributed ones like the red fox (Dunlap et al. 2007). Mercury methylation occurs in aquatic environments and MeHg^+ is absorbed mainly from the gastrointestinal tract of some organisms and then distributes to and accumulates in some organs, including muscle, kidney, liver, and the brain (Bilandzic et al. 2010; Castoldi et al. 2001; Clarkson 1997; Cybulski et al. 2009; Davis et al. 1994; Dehn et al. 2006; Graeme and Pollack 1998; Lopez-Artiguez et al. 1995; Millan et al. 2008; Wren 1986). In contrast to MeHg^+ , elemental Hg is poorly absorbed by the gastrointestinal tract (Wolfe et al. 1998, Wiener et al. 2003) but may be methylated in the rumen of herbivores, absorbed and redistributed (Duffy et al. 2005, Lokken et al. 2009).

The toxicity of MeHg^+ and its accumulation in aquatic biota, humans, and domestic animals is well documented (Abdulla and Chmielnicka 1990; Becker 2000; Brookens et al. 2008; Cybulski et al. 2009; Dunlap et al. 2007; Gaden et al. 2009; Knott et al. 2011b; Lopez-Artiguez et al. 1995). On the other hand, relatively little is understood about these processes in wild terrestrial mammals of the Arctic (Dehn et al. 2006; Gamberg et al. 2005). Studies have shown a correlation of THg concentrations among various tissues (Cumbie 1975; Dip et al. 2001; Kalisinska et al. 2009; Piskorova et al. 2003; Wren 1986). Muscle, liver and kidney are often used as bioindicators to assess biological variation among cohorts, individuals, and populations (Alonso et al. 2004; Becker and Wise 2006; Brookens et al. 2008; Gutleb et al. 1998; McGrew et al. 2013; Yamamoto et al. 1987). Hair is a well documented valuable matrix for some heavy metal contaminant monitoring and is a relatively metabolically inactive tissue that represents a route of elimination (Beckmen et al. 2002; Duffy et al. 2005; Yin et al. 2007). For example, hair is a good indicator of THg in blood as it incorporates MeHg^+ from the blood during growth and has been shown to correlate with levels in the brain (Cerneckiari et al. 1995; Dietz et al. 2009, 2011).

Moreover, the slower decay rates of hair, as well as bone, in the soil make them more readily available than soft tissues for analysis. For example, museum collections often contain samples of bone and pelts. However, few researchers have used bone as a predictor for soft tissue THg concentrations. Interestingly, two reports were found on Hg that was studied in wild carnivore bones, including in the red fox (Lanocha et al. 2012; Millan et al.

2008). Bone is an important tissue because it accumulates some elemental toxicants (Doyle 1979; Halffman 2009; Lehner 2012; Nielsen-Marsh and Hedges 1999). Studies of bone are of great importance for the prevention of diseases caused by trace element imbalance (Klepinger 1984; Kwapulinski et al. 1995; Martinez-Garcia et al. 2005; Takata and Saiki 2004).

The red fox, *Vulpes vulpes*, is a widely distributed, medium-sized canid, which adapts to a variety of environmental conditions (Aubry et al. 2009; Corsolini et al. 1999; Kiener and Zaitsev 2010). Red foxes use a wide trophic niche driven by local and seasonal food availability, including, garbage, small rodents, invertebrates, and small amounts of fruit (Cavallini and Lovari 1991; Ciampalini and Lovari 1985; Corsolini et al. 1999; Doncaster and MacDonald 1991; Goldyn et al. 2003; Hersteinsson and Macdonald 1992; Hewson and Kolb 1975; Jones and Theberge 1982; Kalisinska et al. 2009; Lovari et al. 1994; McIntosh 1963; Saunders et al. 1993; von Schantz 1980). The red fox is an ecological generalist, who sometimes consumes some nutritionally inferior food items (Corsolini et al. 1999, Dell'Arte et al. 2007; Kamler and Ballard 2002).

We evaluated mercury exposure in the wild population of red foxes, *Vulpes vulpes*, of Bethel Alaska. The focus of this paper is to determine if bone and hair can be reliable predictors for THg concentrations in the liver, muscle, renal cortex and renal medulla. In addition this study adds new information on the kidney because the kidney is usually not separated in research. Finally we compare our findings with those of other contaminant research studies using foxes from other northern latitudes.

2 Materials and Methods

2.1 Study Area

The Kuskokwim River (Figure 1), fed by mountain streams which feed into the main river, has been known to have high concentrations of naturally occurring mercury (Rytuba 2003). Also there are several historical mines in the Bethel region. The Red Devil Mine is one in particular that has abandoned tailings which are eroding into the river (Bailey and Gray 1997).

2.2 Carcass collection and processing

A total of 200 red fox, *Vulpes vulpes*, carcasses were donated during the period of November 2010 through February 2011. Trappers anonymously and voluntarily provided carcasses to the Alaska Department of Fish and Game (ADF&G) in Bethel, Alaska. Carcasses remained frozen and were stored outside at the ADF&G office (temperature was continuously below 0°C).

Frozen carcasses were transported to the University of Alaska Fairbanks (UAF) where they were stored at -20 °C. Carcasses were partially thawed and sub-samples of liver, one whole kidney, femur, muscle, and rear paw with fur were collected into Whirl-Pak™ bags and stored at -20 °C or -80°C (femurs). All samples were collected from the right side of the carcass using stainless steel scalpels or scissors. Only foxes with all five available tissues

were analyzed for mercury. Based on these criteria, 65 foxes were used in this study, 35 males and 30 females.

Tissues were prepared for lyophilization according to the following procedures, using disposable stainless steel scalpels (new scalpel for each sample).

2.3 Liver, muscle, renal cortex and renal medulla

Approximately 14 g of frozen liver was cut into small pieces (~1 cm³). The entire quadricep muscle was removed from the femur bone while partially frozen, and cut into small pieces. For dissection of cortex and medulla, kidneys were kept partially frozen on a clean stainless steel tray placed on ice. All instruments used to separate renal cortex and medulla (scalpels and trays) were also kept at -20 °C before use. All tissues were placed into individual pre-weighed Whirl-Pak™ bags and lyophilized for 72 hours (Labconco FreeZone 4.5™ Freeze Dry System). Tissue mass was determined before and after lyophilization.

2.4 Hair and bone

Hair was collected from the right rear paw using a Wahl stainless steel trimmer (carbon blades) and stainless steel scissors. Blades were thoroughly cleaned between each sample. Hair was cleaned and dried following the methods of Castellini et al. (2012).

Bones were cored under a fume hood while wearing a 3M™ particulate respirator N95. The bones were cored using a Dremel™ glass diamond drilling bit, 1/4" (6.350 mm), 663DR, drill speed #23. Each femur was drilled completely through the shaft in three locations to produce six cores (Figure 2). The periosteum and any trabecular bone were removed using a Dremel™ glass diamond taper point sander, 3/32" (2.381 mm), #7144. Total core mass was determined for each individual for a total wet weight (ww). The cores were stored at -20 °C in acid-washed (5% HNO₃) vials prior to degreasing.

Prior to mercury analysis, bone cores were degreased with a series of chloroform treatments in a chemical fume hood (Aerossens et al. 1998; Bell et al. 2001; Dwek 2010; Schutkowski and Herrmann 1999; Zwanziger 1989). Enough chloroform (Chloroform, Reagent ACS, Sciencelab.com) was added to each vial to completely cover bone core samples. Each vial was then loosely capped. Samples were treated for a total of 16 hours, changing chloroform after 8 hours. After chloroform treatments, samples were rinsed several times with ultrapure water. Zwanziger (1989) suggested that freeze drying bone results in decreased THg concentrations, thus degreased samples were air-dried in the fume hood for four days. Bone was homogenized for analysis using a Wig-L-Bug™ (Crescent Dental Company Chicago) with a 9.5 mm stainless steel ball bearing (~ 10 seconds).

2.5 Total mercury (THg) concentration determination

Approximately 16–20 mg of homogenized dry tissue (hair, renal cortex, renal medulla, muscle, liver) was analyzed for THg using a 2-cell DMA-80 Direct Mercury Analyzer (Milestone Inc., Shelton, Connecticut, USA) according to USA EPA method 7473, with a minimum detection limit of 0.037 µg/g (Knott et al. 2011a; Lieske et al. 2011). Approximately 30 mg of homogenized powdered bone was analyzed for THg using a 3-cell

low detection DMA-80 Direct Mercury Analyzer (Milestone Inc., Shelton, Connecticut, USA; USA EPA method 7473. The low detection DMA-80 was calibrated using a 6 point linear calibration curve from 0.25 ng to 6.00 ng; $R^2 = 0.9999$, resulting in a minimum detection limit of 0.008 $\mu\text{g/g}$. All analytical runs included measurement of blanks, liquid standards (1 $\mu\text{g/g}$, 0.1 $\mu\text{g/g}$, 0.01 $\mu\text{g/g}$) and certified reference materials (IAEA-085, IAEA-086, DORM3, DOLT4) depending on tissue (Table I).

2.6 Statistical Analyses

Statistical analyses were performed using the R statistical software package (R Development Core Team, 2011). T-tests were used to compare mean THg concentrations between males and females for all tissues, as well as mean concentration between kidney cortex and kidney medulla. Pearson's correlation statistic was used to estimate the correlation between the two kidney tissues. Linear regression was used to investigate the relationship between hair and bone THg concentrations. Of particular interest was to determine whether hair and/or bone THg concentrations can be used to predict soft tissue, specifically muscle, liver, renal cortex, and renal medulla THg concentrations. Sex was also included as a predictor in all regression models to investigate possible differences in these relationships between males and females.

Models considering hair and bone THg predictors on each soft tissue response were fit separately. Each model first included a term for interaction between sex and THg concentration of hair/bone. If the interaction term was not significant, models were refit without it and the main effects of sex and hair/bone THg were tested. Nonsignificant main effects were then removed, resulting in a final model. Each fitted model was used to compute pointwise 95% prediction intervals for predicting soft tissue THg from hair/bone THg. Significance of all effects was determined using $\alpha = 0.05$.

Residuals from the fitted model were examined to validate model assumptions. A log transformation on the response was employed if residuals showed evidence of nonnormality or nonconstant variance. Standardized residuals having an absolute value greater than three were identified as outliers and removed from the analysis.

R^2 values were used to evaluate the strength and usefulness of models for predicting soft tissue THg concentrations. Model evaluations were based on categories reported by O'Hara et al. (2008), where $R^2 = 0.35$ indicates no meaningful predictive ability, R^2 between 0.36 and 0.55 indicates weakly predictive ability, R^2 between 0.56 and 0.75 indicates moderately predictive ability, and $R^2 = 0.75$ indicates strongly predictive ability.

3 Results

Means $\pm$ standard deviations, minimums, maximums, and medians for each tissue type, as well as summary statistics for each tissue type separated by sex are reported in Table 2. P-values of t-tests comparing mean concentrations between males and females showed no significant differences. A significant difference was found between mean THg concentrations in kidney cortex and kidney medulla (p-value approximately zero). Pearson's correlation between concentration of kidney cortex and kidney medulla was 0.813.

Regression models found no significant interactions between sex and hair/bone THg concentrations for any of the soft tissues. The hair/bone main effects were significant in each regression model, indicating that hair/bone THg concentrations can be used to predict soft tissue THg concentrations. The main effect for sex was not significant in any of the regression models, suggesting that the relationship between soft tissue THg concentrations and hair/bone THg concentrations is the same for male and female foxes. Results of regression analyses are shown in Table 3. Fitted regression models with 95% prediction intervals are displayed in Figures 3 and 4.

Hair THg concentration is a moderate predictor of renal cortex ($R^2 = 0.61$) and liver ($R^2 = 0.63$) THg concentration. Hair THg concentration is weakly predictive of renal medulla ($R^2 = 0.50$) and muscle ($R^2 = 0.39$) THg concentrations. Bone THg concentration has no meaningful predictive value for liver ($R^2 = 0.28$), renal cortex ($R^2 = 0.33$), or renal medulla ($R^2 = 0.29$) THg concentrations. Bone THg concentration is weakly predictive of muscle ($R^2 = 0.36$) THg concentration.

In the regression analysis of both hair and bone, two outliers were identified and removed. However, the removal of these outliers did not change the results of regression models. Responses for models using bone THg concentration as a predictor were log transformed due to evidence of nonconstant variance.

5 Discussion

Many studies have focused on characterizing mercury levels in fox populations and establishing differences between sex and age groups, e.g. Kiener and Zaitsev (2010) and Lanocha et al. (2014). Mercury concentrations in terrestrial mammals occasionally show a wide intraspecific variability (Duffy et al. 2005; Kalisinska et al. 2009, 2012) influenced by natural environmental differences. Mercury concentrations in livers from red foxes, *Vulpes vulpes*, from the Province of Siena in Italy were shown to differ in relation to sex and age (Corsolini et al. 1999). The difference in hepatic THg concentrations between foxes from Italy and Alaska based on a geochemical background is ~0.16 vs ~1.20 mg/kg dw. Their study differed from ours in regards to sex and age of the fox. We did not consider age in our study as we had very few foxes older than two. In addition, our study found no significant difference in male and female liver mean THg concentrations (males are 1.261 mg/kg dw; females are 1.185 mg/kg dw) as compared to Corsolini et al. (1999) study (yearling males avg. 0.18 mg/kg dw and adult males avg. 0.13 mg/kg dw; yearling females avg. 0.14 mg/kg dw and adult females avg. 0.16 mg/kg dw). The higher THg concentrations in liver of Alaska foxes may indicate they are eating at a higher trophic level, as Dehn et al. (2006) showed a similar result with polar bears, (*Ursus maritimus*); stable isotope studies could support this.

In Poland, Cybulski et al. (2009) assessed mercury levels in livers and kidneys of *Vulpes vulpes*. The outer portion of the kidney which consists of the nephrons, is the cortex and the inner portion which consists of the pyramids, is the medulla (Boundless 2014). Nephrons, the functional structure in the kidney, filter the blood to regulate chemical concentrations to produce the urine (Boundless 2014). After this initial filtering, the waste passes deep into

medullary pyramids producing urine to be passed to the bladder (Boundless 2014). Their study differed from ours in that they studied foxes from a fur fox farm where our foxes were from their natural habitat. Cybulski and colleagues reported liver Hg concentrations as 0.257 ± 0.3403 mg/kg ww and kidneys as 0.600 ± 1.1112 mg/kg ww. Their research also used whole kidney where our research sub-sampled the renal cortex and renal medulla. We did not use whole kidney in our analysis as it may contain other contaminants such as blood and the waste products from the blood, which may interfere with THg analysis. Our study reported the average THg concentrations for all red fox livers analyzed as 1.226 mg/kg ww, renal cortex having 2.122 mg/kg ww, and renal medulla having 1.177 mg/kg ww, which are higher than that of the fox fur farm.

Samples of muscles, kidneys, and livers of red foxes, *Vulpes vulpes*, were collected in Central region of Slovak Republic, an emissions contaminated area (Piskorova et al. 2003). This study indicated mercury levels between 0.06 and 1.43 mg/kg, in the tissues from wild foxes. In our study, our values are similar to their upper range. The muscle we used was the quadracep in contrast to their *m. semimebranosus* muscle. In addition, their numbers of samples were much smaller than in our study. We used 65 samples in contrast to their samples of 12 kidneys, eight livers and one muscle. Another difference is the use of whole kidney in the Piskorova et al. (2003) study, while we divided the kidney into renal cortex and renal medulla. Their study showed THg concentration level averages in liver as 0.22 mg/kg, kidney as 0.63 mg/kg, and muscle as 0.013 mg/kg.

In another study of the red fox, *Vulpes vulpes*, the renal cortex, liver, and muscle was analyzed for mercury concentrations to determine if these mercury levels can be used as a bioindicator of toxic metal accumulation in urbanized habitats in Croatia (Bilandzic et al. 2010). Their results found that THg concentration values were slightly higher in rural areas of Croatia. This was the only study found, to date, that used the renal cortex and not the entire kidney. Their samples sizes were again small, 12 red fox for the suburban study and 16 red fox for the rural study. Their THg concentration averages for suburban and rural red fox is as follows: muscle (name not reported) 0.007 mg/kg ww and 0.004 mg/kg ww, liver 0.025 mg/kg ww and 0.009 mg/kg ww, and renal cortex 0.064 ug/g ww and 0.032 ug/g ww, respectively. Table 2 reports our dry weight data and suggests the rural Alaska red fox have higher THg concentrations in their tissue than the Croatia foxes in Bilandzic et al.'s (2010) study.

Two European studies reported on THg concentrations that were found in wild red fox bones (Lanocha et al. 2012; Millan et al. 2008). In the compact bone tissue reported, the results showed 0.0054 mg/kg dw (ranges 0.0012 – 0.0226) and 0.012 mg/kg dw (ranges nd – 0.038) respectively (Lanocha et al. 2012 and Millan et al. 2008). This is in the same range as our Alaskan red foxes reporting mean THg for compact bone as 0.004 mg/kg with a range of 0.001 to 0.010 mg/kg (Table 2).

In general, with exception of farmed foxes, the wild red foxes of Alaska, living in a relatively pristine watershed ecosystem, have higher natural THg concentration levels in their tissues than those reported in highly populated Europe where the environment is

patchy. The more rural and intact natural environment of rural Alaska may allow the red fox to feed higher on the food chain, with an abundance of fish and small mammals.

6 Conclusion

This is the first data set reported for the red fox in Alaska in which THg concentrations for several tissues from the same animal were compared. Our findings support the hypothesis, in part, that THg concentration in hair is correlated across tissues and these correlations may be strong enough so one can predict THg concentrations in some tissues, specifically skeletal muscle, renal medulla, renal cortex, and liver. THg concentration in hair had a significant linear relationship to THg concentration in liver, renal cortex, renal medulla, and muscle for both males and females. Since hair is a good matrix to predict THg concentration in the renal cortex and liver for red foxes, hair samples from foxes could be used to indicate approximate liver and renal THg concentrations.

Acknowledgments

We would like to thank all the trappers that submitted carcasses to ADF&G; and Dr. Kimberlee Beckmen for assistance with sampling. We would like to thank Dr. John Blake and assistants at the UAF Biological Research and Diagnostics Facility (BiRD) for the use of their necropsy suite for the collection of samples. I would also like to thank Megan Templeton for her help in coring the bones. Many thanks to Dr. Stephen Long of NIST for his efforts in working with the author to be able to certify bone meal (1486) for use as a THg SRM. Funding provided by the Alaska INBRE program. Research reported in this publication was supported by the National Institute of General Medical Sciences of the National Institutes of Health under Award Number P20GM103395.

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Highlights

- For the red fox hair THg concentration is highly correlated to kidney, liver and muscle tissues
- For the red fox, bone THg concentration is weakly predictive of muscle
- The red fox can serve as a sentinel species in a changing climate for heavy metals such as mercury in monitoring contaminants bioavailability in the Circumpolar North

Figure 1.
Bethel Alaska and The Kuskokwim River

Figure 2.
Arrows showing where three cores were drilled through femur.

Figure 3. Fitted Regression Models for Hair THg mg/kg (x-axis) vs each soft tissue THg mg/kg (y-axis). Models fitted with 95% pointwise prediction intervals. Clockwise from top left: hair vs. liver, hair vs. muscle, hair vs. renal medulla, hair vs. renal cortex.

Figure 4. Regression Models with Bone THg mg/kg (x-axis) vs each soft tissue THg mg/kg (y-axis). Models fitted with 95% pointwise prediction intervals. Clockwise from top left: bone vs. liver, bone vs. muscle, bone vs. renal medulla, bone vs. renal cortex.

Table 1

Percent recovery of liquid standards and certified reference materials

	% Recovery				
	Bone	Hair	Liver	Renal Cortex & Medulla	Muscle
Liquid Standard ^a (mg/kg)					
0.010	0.010				
0.100		0.097	0.099	0.096	0.103
1.000		0.987		0.964	1.043
Reference Materials (g)					
SRM 1486 ^b (0.0300)	109.75				
IAEA 085 ^c (0.0200)		101.43			
IAEA 086 ^c (0.0200)		106.32			
DORM3 ^d (0.0100)			107.95	102.60	110.07
DOLT4 ^e (0.0100)			110.50	97.83	109.62

^a Liquid Standards: 0.010 in 3.7% HCL [9.913 ppb] for bone; 0.100 in 3.7% HCL [99.9 ppb], 1.000 in 3.7% HCL [999 ppm] for hair and renal cortex/medulla; 0.100 in 3.7% HCL [100.52 ppb] for liver; 0.100 in 3.7% HCL [99.02] for muscle.

^b Bone meal SRM 1486 (note: Author worked with Dr. Stephen Long of the National Institute of Standards and Technology. This SRM will now be assigned a reference value on the certificate)

^c Hair certified standard; IAEA 085 = 23.2 µg/g; IAEA 086 = 0.573 µg/g; International Atomic Energy Agency

^d Fish protein certified standard; 0.382 ± 0.060 µg/g; National Research Council of Canada

^e Dogfish liver certified standard; 2.54 ± 0.22 µg/g; National Research Council of Canada

Table 2

Mean±SD, minimum, maximum, and median mercury concentration, for each tissue type combining male and female red fox and separated by sex.

Tissue	Mean ± SD	Minimum	Maximum	Median
Bone	0.004 ± 0.002	0.001	0.010	0.004
Hair	2.580 ± 1.959	0.429	9.604	2.024
Muscle	0.567 ± 0.464	0.097	2.001	0.432
Liver	1.226 ± 1.133	0.114	6.149	0.924
Renal Medulla	1.177 ± 0.968	0.217	4.061	0.754
Renal Cortex	2.122 ± 1.486	0.429	6.049	1.929
Bone				
Male	0.005 ± 0.002	0.001	0.010	0.004
Female	0.004 ± 0.002	0.001	0.010	0.004
Hair				
Male	2.794 ± 2.188	0.429	9.604	2.222
Female	2.320 ± 1.666	0.539	5.926	1.705
Muscle				
Male	0.608 ± 0.441	0.097	1.932	0.510
Female	0.519 ± 0.494	0.114	2.001	0.341
Liver				
Male	1.261 ± 1.101	0.225	6.149	0.969
Female	1.185 ± 1.188	0.114	5.334	0.887
Renal Medulla				
Male	1.307 ± 0.968	0.217	3.794	0.996
Female	1.026 ± 0.961	0.256	4.061	0.632
Renal Cortex				
Male	2.341 ± 1.575	0.429	6.049	2.037
Female	1.867 ± 1.356	0.509	5.621	1.340

Reported in mg/kg dw

R² and p-values for testing association between hair and bone vs soft tissues (no sex or interaction) ^{*}.

Table 3

		Renal Cortex	Renal Medulla	Liver	Muscle
Hair	R ²	0.6073	0.5003	0.6268	0.3886
	p-value	3.34e-14	4.5-7e-11	6.804e-15	2.383e-07
Bone	R ²	0.3287	0.2868	0.2768	0.3595
	p-value	5.997e-07	4.293e-06	6.76e-05	1.31e-07

^{*} Responses for bone were log transformed