Science of the Total Environment 612 (2018) 1559–1567

Contents lists available at ScienceDirect

Science of the Total Environment

journal homepage: www.elsevier.com/locate/scitotenv

Chronic arsenicosis and cadmium exposure in wild snowshoe hares (*Lepus americanus*) breeding near Yellowknife, Northwest Territories (Canada), part 2: Manifestation of bone abnormalities and osteoporosis

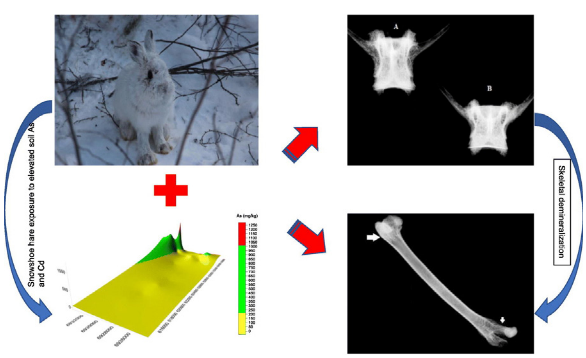
Amuno S. [a,*,1], Al Kaissi A. [b], Jamwal A. [c], Niyogi S. [c,d], Quenneville C.E. [e,f]

[a] School of Environment and Sustainability, University of Saskatchewan, Saskatoon, Canada
[b] Ludwig Boltzmann Institute of Osteology-Hanusch Hospital, First Medical Department and Orthopedic Hospital of Speising, Vienna, Austria
[c] Department of Biology, University of Saskatchewan, Saskatoon, Canada
[d] Toxicology Centre, University of Saskatchewan, Saskatoon, Canada
[e] Department of Mechanical Engineering, McMaster University, Ontario, Canada
[f] School of Biomedical Engineering, McMaster University, Ontario, Canada

HIGHLIGHTS

- Skeletal pathology was studied in wild hares from the Giant mine area and reference location.
- Vertebrae from the mine area also showed relatively lower bone area and tissue area compared to the reference location.
- Osteoporosis, cortical fractures, sclerosis, and cyst like changes were observed in hares from the two groups.
- Bone stiffness and peak load tended to be relatively reduced in specimens from the mine area compared to the reference site.
- As and Cd may be involved in the etiology of bone damage in exposed snowshoe hare population.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 3 July 2017
Received in revised form 28 August 2017
Accepted 28 August 2017
Available online xxxx

Editor: F.M. Tack

Keywords:
Chronic arsenicosis
Osteoporosis
Arsenic contamination
Snowshoe hares

ABSTRACT

Various bone abnormalities, including osteoporosis, have been associated with chronic arsenic and cadmium exposure in experimental animal models, but information regarding the bone pathology of wild population of small mammals breeding in contaminated environment is limited. This present study was conducted to comparatively assess the prevalence and pattern of skeletal abnormalities in free ranging snowshoe hares inhabiting an area heavily contaminated by arsenic and other trace metals, near the vicinity of the abandoned Giant mine, and in a reference location approximately 20 km from the city of Yellowknife, Northwest Territories, Canada. The femur and vertebrae of snowshoe hares from the mine area and reference location were subjected to bone densitometry examination and biomechanical testing using dual energy X-ray absorptiometry (DXA) and 3-point bending test. *t*-test results indicated that femoral densitometry parameters such as bone mineral density (BMD) ($p = 0.5$), bone mineral content (BMC) ($p = 0.675$), bone area (BA) ($p = 0.978$) and tissue area (TA) ($p = 0.549$) were not significantly different between locations. All densitometry parameters of the vertebrae (BMD, BA and TA) differed between locations ($p < 0.05$), except for BMC ($p = 0.951$) which showed no significant

* Corresponding author.
E-mail address: soa882@mail.usask.ca (S. Amuno).

[1] Note: Dr. Amuno is an adjunct professor at the School of Environment and Sustainability, and his participation in this study was undertaken independently and apart from his current work with the Nunavut Impact Review Board (NIRB). The analysis and views expressed in the study remain solely those of the authors and do not constitute the views of NIRB.

http://dx.doi.org/10.1016/j.scitotenv.2017.08.280

difference between the two locations. Vertebrae from the mine area also showed relatively lower BA and TA compared to the reference location. A constellation of skeletal abnormalities were also observed along the axial and appendicular bones respectively. Specifically, growth defects, osteoporosis, cortical fractures, sclerosis, and cyst like changes were commonly observed in the femurs and vertebrae of hares from both locations. With respect to biomechanical properties, only bone stiffness and peak load tended to be relatively reduced in specimens from the mine area, whereas work to failure was notably increased in specimens from the reference site compared to those from the mine area. Taken together, the results of this preliminary study suggest that chronic concomitant exposure to arsenic and cadmium may be involved in the etiology of various bone abnormalities, including osteoporosis in wild population of snowshoe hares from the Yellowknife area. The result presented in this study represent the first evaluation of osteological effects in free-ranging furbearers (snowshoe hares) diagnosed with arsenicosis, and concomitantly exposed to environmental levels of cadmium.

1. Introduction

Furbearer species such as snowshoe hares, with limited home range are known to inhabit different areas around Yellowknife, but information regarding their physiological condition, particularly bone health is unknown. Recognizing that elevated levels of arsenic (As) and other toxic trace metals such as cadmium (Cd) have been reported in the terrestrial ecosystem of the Yellowknife area for several decades, there are growing concerns that uptake, and concomitant environmental exposure to these elements may induce adverse health effects, including bone deterioration in exposed wildlife species in the area (Koch et al., 2005; Saunders et al., 2011). While a growing number of studies have already identified the skeletal system as a target site for As and Cd toxicity, there is urgent need to monitor whether current exposure levels induces osteological abnormalities in exposed terrestrial wildlife from the Yellowknife area (Brzóska and Moniuszko-Jakoniuk, 2004; Kido, 2013; Lim et al., 2016). Considering that damage to bone is known to impair mobility and reduce fitness in animals, and may result in the inability of animals to mate and escape predation, scientific investigation monitoring the incidence and prevalence of bone abnormalities in animals from contaminated environment may be useful for developing an increased understanding of bone pathology of animals under chronic environmental exposure. As bone is a highly mineralized connective tissue, consisting of hydroxyapatite and collagens, there are growing concerns that its properties and function may be impaired by the combined influence of As and Cd toxicities (Florencio-Silva et al., 2015; Brzóska and Moniuszko-Jakoniuk, 2005). For example, Cd exposure is known to alter bone quality and increase the risk of osteoporosis in both human subjects and animal models, whereas As has been shown to exert its influence on bone mineralization by substituting for phosphate in the hydroxyapatite crystal, which may also disrupt osteoclast and osteoblast activities (Odstrcil et al., 2010; Hu et al., 2012; Akbal et al., 2014). As previous terrestrial monitoring studies in the Yellowknife area have focused on measuring the levels of As in surface soils, vegetation, small mammals and birds (Koch et al., 2000; Saunders et al., 2011; Hocking et al., 1978), there is need to start documenting the prevalence and pattern of skeletal abnormalities in wildlife species from As-contaminated areas in order to evaluate the severities of exposure-related effects. The specific aim of this study was twofold. The first was to comparatively assess the prevalence and pattern of skeletal abnormalities in wild snowshoe hares breeding in an area heavily contaminated by As including Cd, near the vicinity of the abandoned Giant mine, and in reference location outside the city of Yellowknife, in order to ascertain whether there is difference between the two groups. The second was to comparatively assess the effects of chronic concomitant exposure to As and Cd on bone health, including bone mineral density parameters and biomechanical properties of snowshoe hares from the two groups, in order to determine whether there is a difference between the two groups. The overall objective of this study was to determine whether concomitant environmental exposure to As and Cd may be associated with various skeletal abnormalities, and or increase the risk of osteoporosis in exposed snowshoe hares from the study area.

2. Historical perspective of As contamination of the study area

Gold was discovered in Yellowknife in 1936, and by 1938 the City of Yellowknife was already an active gold mining community (Environmental Sciences Group, 2001). The occurrence of gold mineralization in the Yellowknife area is mostly associated with arsenopyrite and pyrite, and as a result three gold mines operated in the area under various owners during different timescales such as Con mine (1938–2003), Negus mine (1939–1952) and Giant mine (1948–2004). The Giant mine was the most prominent gold producer in the region. The Giant mine deposits occur within the Yellowknife Greenstone Belt, and mineralization in the south and central areas of Giant mine was identifiable as broad zones of silicification accompanied by quartz-carbonate veins with sulphide mineralization, which are typically bounded by sericite to chlorite schist (Government of Northwest Territories, 2016). In the north of the mine, gold was located within shallow dipping shear zones in relatively narrow composite quartz-carbonate veins. The quartz veins contain pyrite, arsenopyrite, sphalerite, chalcopyrite, stibnite, sulphosalts, and pyrrhotite (Government of Northwest Territories, 2016). Due to its geology, background levels of arsenic in soils around the vicinity of the Giant mine area, and surrounding locations are elevated, and there is evidence to suggest that previous gold mining activities at different times also contributed to the increased levels of As in some areas in Yellowknife (Environmental Sciences Group, 2001). For example, as a result of the roasting of arsenopyrite bearing gold ores, and the poor emission control standards at the Giant mine stack during the early phase of Giant mine operations, an estimated 20,000 tonnes of roaster generated arsenic trioxide was released into the surrounding environment causing contamination of both the terrestrial and aquatic ecosystems (Bromstad et al., 2017). Despite the concerns that As exposure is associated with different adverse health effects in exposed wildlife populations (Eisler, 1994), no attempt has been made to specifically investigate exposure-related effects, including osteological anomalies in terrestrial wildlife species from the area.

3. Material and methods

A wildlife research permit (WL500454) and ethical clearance for wildlife handling was obtained from the Department of Environment and Natural Resources, Government of the Northwest Territories. A general research licence (No. 15924) was also obtained from the Aurora Research Institute prior to commencement of the field study. An experienced local furbearer hunter was employed to assist in determining breeding locations of snowshoe hares prior to trapping in the study area. A total of 14 sub-adult snowshoe hares aged approximately 1 to 2 years old were trapped between 0 and 3 km radius of the abandoned

Giant mine, and compared to 14 sub-adult control animals captured approximately 20 km from the mine area. Trapping of animals took place between October and November 2016. Animals were euthanized by cervical dislocation by an experienced hunter, and femur and vertebrae were harvested from each animal and preserved in ethanol during shipment, and prior to laboratory testing. Animals were not segmented into gender due to the unequal numbers of male and female hares captured during field work, in order to ease data interpretation. Environmental contamination of the study area, was determined through measurement of total As and Cd in willows, soils, whereas evidence of chronic arsenicosis and Cd exposure in hares was determined through estimating the accumulation levels of As and Cd in bones, nails, liver, kidney and stomach content of hares (Refer to Part 1 paper for complete data).[2]

3.1. Radiographic analysis and densitometric measurements

Radiographic assessment of femur and vertebrae of snowshoe hares was conducted at the Centre for Bone and Periodontal Research, McGill University, Quebec Canada. A single X-ray of femur and vertebrae samples was taken using a Kubtec Xpert 80 radiography system with automatic calibration. Densitometric measurement was also obtained using an animal densitometer (Luna Piximus) for assessment of bone mineral density. Due of the sizes of femurs, only mid-shaft of femurs was included in the region of interest (ROI) as shown in Fig. 1a (the green rectangle). The vertebrae were measured in whole. The results were provided in terms of bone mineral density (BMD) (g/cm^2), bone mineral content (BMC) (g), bone area (BA) (cm^2) and tissue area (TA) (cm^2).

3.2. Biomechanical testing

Biomechanical measurements of the femurs were conducted at McMaster University, Hamilton, Ontario, Canada. All of the samples were immersed in a saline solution at 4 °C for 48 h prior to biomechanical testing, and allowed to warm to room temperature for a minimum of one hour prior to testing. Key measurements were taken of each femur using calipers. These included overall length (from intercondylar notch to femoral head), and width in two directions (anterior-posterior and medial- lateral) at mid-diaphysis (defined as half the overall length). Femurs were also weighed using a digital scale. Each femur was tested in three-point bending using a material testing machine (Electropuls 1000, Instron, Canton MA). The bottom supports were 5 cm apart, and the mid-diaphysis mark was aligned with the center load application point (Fig. 1b). Ramp loads were applied at 1 mm/min from 2 to 50 N and unloaded at the same rate eight times, with the first five serving to relax the specimen and the final three being used for stiffness calculations. Then the femur was ramp loaded at the same rate to failure. Output measures were stiffness, yield load, peak load, and work to failure, calculated as the area under the force-deflection curve during the failure test (Fig. 2).

3.3. Statistical analysis

The assumptions of normality of distribution, and homogeneity of variances were verified with Shapiro-Wilk and Levene's test, respectively. Statistical comparisons were made by independent *t*-test (SPSS software, version 16.0). A *p*-value of ≤0.05 was considered to be statistically significant.

2 Complete data for arsenic and cadmium levels in the natural environmental (i.e. soils and willows), including tissues (livers, kidney, stomach content, bones and nail) of snowshoe hares are available in the Part 1 publication, as such the result were not duplicated in this current paper. Evidence of chronic arsenicosis in snowshoe hares from the Giant mine area, including higher Cd exposure has been presented in Part 1.

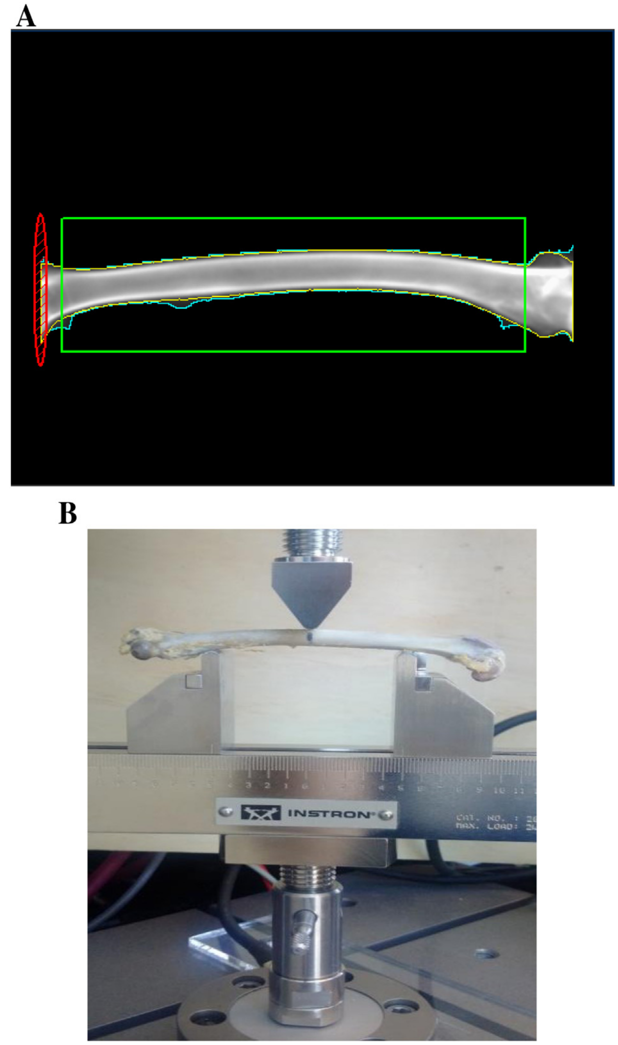


Fig. 1. a: Delineation of the region of interest (ROI) for bone densitometry measurement b: Test setup of a femur sample placed on a three-point bending jig, with mid-diaphysis (marked) aligned with the load application point, and 5 cm between bottom supports.

4. Results and discussion

4.1. Osteodensitometry changes and trace element accumulation in snowshoe hares from Yellowknife

The result of bone densitometric measurement of snowshoe hares is presented in Table 1. An independent sample *t*-test ($\alpha = 0.05$) was further conducted to compare the statistical differences between the two locations, with respect to bone mineral density parameters. *t*-Test revealed no significant differences between the two groups with respect to bone densitometric parameters ($p > 0.05$), even though our preliminary results already confirmed that hares from the mine area showed a relatively higher level of arsenic concentration in nail clippings and organ tissues compared to those from the reference site. Preliminary data indicate that in general, the study area show a pattern of anomalous concentration of As exceeding 150 mg/kg for soils, and in some locations As concentration in willows reached up to 59.4 mg/kg especially near the vicinity of the Giant Mine site, but tended to show a decrease concentration away from the mine site. The maximum concentration of As noted in the soil from the mine area exceeded 1500 mg/kg. Cd levels near the mine area were generally in the range of 0.2 mg/kg to 1.2 mg/kg for soils, and reached up to the 2.39 mg/kg in willows, but tended to decrease away from the mine area (Refer to Part 1 publication for full data). Parameters for diagnosing chronic arsenicosis including

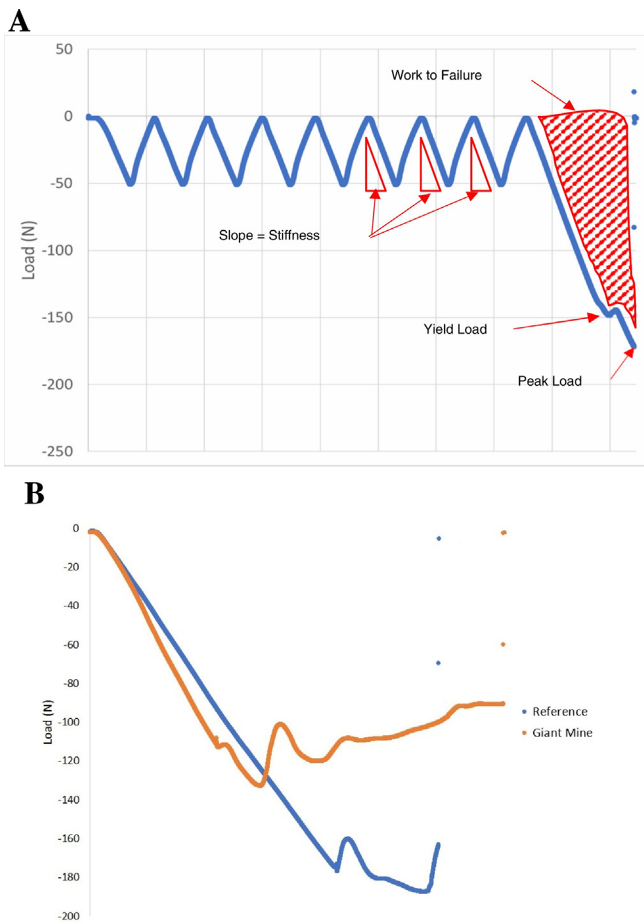


Fig. 2. a: A sample load curve, each specimen was cycled from 2 to 50 N in three-point bending (compression being negative load) eight times, followed by a constant displacement ramp load to failure. The slope of the line for the last three cycles was calculated for stiffness, and the yield load, peak load, and work to failure were identified as shown. b: Representative load curves of work to failures of snowshoe hares bones from two locations.

Cd exposure in hares included the data for total As and Cd in nails and other organ tissues (complete data available in the Part 1 publication). To summarize the accumulation data, As levels in nails of hares from the reference site ranged from 0.047 μg/g to 0.936 μg/g, while those from the mine area ranged from 1.087 μg/g to 4 μg/g. In addition, Cd concentration in the nails of hares from the mine area ranged from 0.007 μg/g to 0.476 μg/g, whereas those from the reference site ranged from 0.003 μg/g to 0.027 μg/g. As was generally below detection limit in all the bone specimens, except for three bone samples from the Giant mine area which showed detectable levels of As. Cd was noted to be in the range of 0.006 μg/g to 0.013 μg/g in bones of hares from the reference site, while that from the mine area was 0.012 μg/g to 0.053 μg/g. Similarly, both hepatic and renal concentration of As and Cd were also noted to be generally elevated in hares from the mine area compared to the reference site. The relatively higher concentration of As and Cd in nails of hares is suggestive of chronic exposure, and this formed the basis for studying ostological abmormalities, including bone demineralizing effects in the exposed animals.

In the femurs examined, BMD ranged from 0.27 g/cm^2 to 0.31 g/cm^2 in specimens from the Giant mine area, while that from the reference site was between 0.21 g/cm^2 to 0.33 g/cm^2. BMC ranged 1.40 g to 1.72 g for specimens from the mine area, while that from the reference site was 1.1 g to 1.99 g. BA ranged from 5.08 cm^2 to 5.97cm^2 in specimens from the mine area, while that from the reference site was 5.04 cm^2 to 6.34cm^2. TA ranged from 5.27 cm^2 to 6.04 cm^2 in specimens from the mine area, while that from the reference area 5.34cm^2 to 6.4cm^2. *t*-Test results indicated that femoral densitometry parameters such as BMD ($p = 0.5$), BMC ($p = 0.675$), BA ($p = 0.978$) and TA ($p = 0.549$) were significantly not different between locations. In the vertebrae examined, BMD ranged from 0.24 g/cm^2 to 0.29 g/cm^2 in specimens from the mine area, while that from the reference site was 0.16 g/cm^2 to 0.266 g/cm^2. BMC varied from 0.53 g to 0.76 g in specimens from the mine area, while that from the reference area ranged from 0.36 g to 0.78 g. BA ranged from 1.91 g to 2.63 g in specimens from the mine area, while that from the reference site was 2.25 g to 3.42 g. TA ranged from 2.07 g to 2.93 g in specimens from the mine area, while that from the reference site was 2.66 g to 4.22 g. *t*-Test results revealed that some vertebrae densitometry parameters such as BMD ($p = 0.007$) and BA ($p = 0.039$) were significantly different between locations, except for BMC ($p = 0.951$), which was not statistically significant between the two groups. Further, TA exhibited a certain trend toward significance ($p = 0.08$), although in general, vertebral specimens from the mine area tended to exhibit reduced BA and TA compared to those from the reference site. The observed reduction in BA and TA may be indicative of reduced bone formation and increased bone resorption caused by contaminant exposure, subsequently causing the vertebrae to demineralize. The BMD changes noted in the specimens may be indicative of the combined effects of As and Cd in inducing skeletal demineralization, subsequently causing changes in bone mineral density. (Odstrcil et al., 2010; Hu et al., 2012; Brzóska and Moniuszko-Jakoniuk, 2005).

4.2. Biomechanical properties of femoral bones of snowshoe hare from Yellowknife

Several biomechanical parameters were measured for the two groups (Tables 2 and 3). No statistical differences were found between the groups for length ($p = 0.64$), width 1 ($p = 0.68$), width 2 ($p = 0.10$), or weight ($p = 0.18$). The average stiffness in specimens from the reference site was 254.6 N/mm. The load-deflection curves during the three stiffness trials were highly linear, with an average R^2 of 0.999 (minimum 0.995). In addition, the average yield load and average peak load of bone samples were 159.6 N and 179.8 N, respectively. The average work to failure was 96.7 Nmm. For the mine area, the average stiffness of bones was 244.4 N/mm. The load-deflection curves during the three stiffness trials were also highly linear, with an average R^2 of 0.999 (minimum 0.997). The average yield load and average peak load of bone samples from this area was 162.6 N and 169.6 N, respectively. Average work to failure was 129.3 Nmm. Yield loads and

Table 1
Osteodensitometry parameters of Snowshoe hares from Giant mine and Reference sites.

Locations	Giant mine area (minimum–maximum)		Reference site (minimum–maximum)	
	Femurs ($n = 7$)	Vertebrae ($n = 7$)	Femurs ($n = 7$)	Vertebrae ($n = 5$)
Bone mineral density (g/cm^2)	0.27–0.31	0.24–0.29	0.21–0.33	0.16–0.266
Bone mineral content (g)	1.40–1.72	0.53–0.76	1.1–1.99	0.36–0.78
Bone area (cm^2)	5.08–5.97	1.91–2.63	5.04–6.34	2.25–3.42
Tissue area (cm^2)	5.27–6.04	2.07–2.93	5.34–6.4	2.66–4.22

Table 2
Measurements of length, width and weight of femurs of snowshoe hares from Giant mine and Reference site.

Specimen	Reference site				Giant mine area			
	Length (mm)	Width 1 (mm)	Width 2 (mm)	Weight (g)	Length (mm)	Width 1 (mm)	Width 2 (mm)	Weight (g)
A	101.8	6.1	6.7	7	96	5.8	6.9	7
B	96.1	4.9	5.7	6	95.1	5.3	6.1	6
C	101.1	5.9	6.1	6	100.6	5.8	6.9	7
D	88.6	5.3	5.7	6	103.4	5.7	6.2	7
E	99	6.1	6.4	8	95.5	6	6.9	6
F	100	5.8	6.5	8	100.7	5.7	6.6	8
G	103	6.1	6.9	8	85.8	4.8	5.6	5
H	99.8	5.3	5.9	7	101.4	5.9	7.2	7
I	98.8	5.7	6.5	8	103.3	5.2	6.2	7
J	99.6	5.8	6.1	8	95	5.9	6.3	7
K	94.7	5.2	6.1	7	96.1	5.4	6.8	7
L	92.7	5.5	6.1	6	92.7	5.5	6.2	6
M	93.8	5.7	6.1	6	91.9	5.4	6.2	6
N	95.7	5.1	6.1	7	95.8	5.3	6.4	6
Average	97.5	5.6	6.2	7.0	96.7	5.6	6.5	6.6
Standard deviation	4.0	0.4	0.4	0.9	4.9	0.3	0.4	0.8

peak loads of the bones were also not significantly different between locations ($p = 0.82$; $p = 0.54$).

Stiffness is the measure of resistance to force, and the stiffer a bone is the more force it requires to produce a given a displacement (Turner, 2006). In our study, bone stiffness tended to be somewhat higher in snowshoe hares from the reference site compared to those from the mine area, but this was not significantly different between locations ($p = 0.64$). It is likely that the relatively higher bone stiffness of the specimen may have been influenced by increase in bone mineralization, which may in turn adversely affect the structural rigidity of the bones causing bone brittleness and may increase the risk of fractures. Given that the average bone stiffness were not significantly different between the two locations, it is likely that the bones from both areas may be similarly at risk of fractures. Work to failure represents the amount of work that must be done to fracture the bone (Turner, 2002). In our study, work to failure was not statistically significant ($p = 0.082$) between the two groups, but this was approaching the level of significance. As indicated in Table 3, the average work to failures (standard deviation) for each group were 96.7 (43.7) Nmm for reference site, and 129.3 (51.3) Nmm for the Giant mine site. As a result of the fairly large standard deviations, no statistical conclusions could be drawn from the data, but there was a strong tendency toward the bones from the mine area having greater work to failure. The work to failures in both locations was further illustrated in Fig. 2b, which showed the representative load curves for bone samples from the two locations. While on average the two locations had very similar stiffnesses and yield loads, the samples from the Giant Mine tended to undergo a greater amount of plastic deformation prior to ultimate failure. This resulted in the higher trend of the Giant Mine work to failure specimens.

Yield loads and peak loads were not significantly different between locations ($p = 0.82$; $p = 0.54$). Previous investigations have shown that chronic exposure to low concentrations of Cd can alter the mineral status and bone mechanical properties, thereby increasing the risk of osteoporosis and fractures in rodent models (Brzóska and Moniuszko-Jakoniuk, 2004; Brzóska and Moniuszko-Jakoniuk, 2005).

4.3. Radiographic assessment of femoral bones and vertebrae of snowshoe hares from Yellowknife

A radiographic analysis of the femur and vertebrae of snowshoe hares was conducted. For each location, 7 femurs and 5 vertebrae were randomly selected and examined for bone abnormalities. Radiographic assessment of bones from the mine area and reference location is described below:

4.4. Reference area

In the control location, bone specimens exhibited osteoporosis and avulsion cortical fracture (avulsion fracture is defined as separation of a small fragment of bone cortex at the site of attachment of a ligament

Table 3
Biomechanical properties of femurs of snowshoe hares from Giant mine and Reference site.

Specimen	Reference site				Giant mine area			
	Stiffness (N/mm)	Yield Load (N)	Peak Load (N)	Work to Failure (Nmm)	Stiffness (N/mm)	Yield Load (N)	Peak Load (N)	Work to Failure (Nmm)
A	350.9	197.6	256.4	136	186.0	159	159	79
B	331.5	174.6	229.5	145	206.6	111	111	107
C	169.2	137.6	149.9	157	251.5	116	133	129
D	210.9	176.7	187.3	99	284.0	159	159	116
E	336.7	192.1	259.8	110	267.0	224	224	201
F	158.2	93.6	109.7	46	263.6	159	170	167
G	239.2	166.6	179.7	23	112.7	98	98	83
H	275.4	156.9	156.9	124	287.5	125	163	98
I	287.8	118	118	155	240.0	183	183	160
J	227.2	169	189.5	70	334.1	237	268	246
K	252.5	160.7	173.8	85	252.0	185	185	164
L	290.4	189.2	189.2	55	242.8	175	175	81
M	198.6	146.8	154.3	48	229.5	186	186	85
N	235.5	155.1	162.7	101	265.1	160	160	94
Average	254.6	159.6	179.8	96.7	244.4	162.6	169.6	129.3
Standard deviation	60.6	28.8	44.8	43.7	52.1	40.6	42.6	51.3

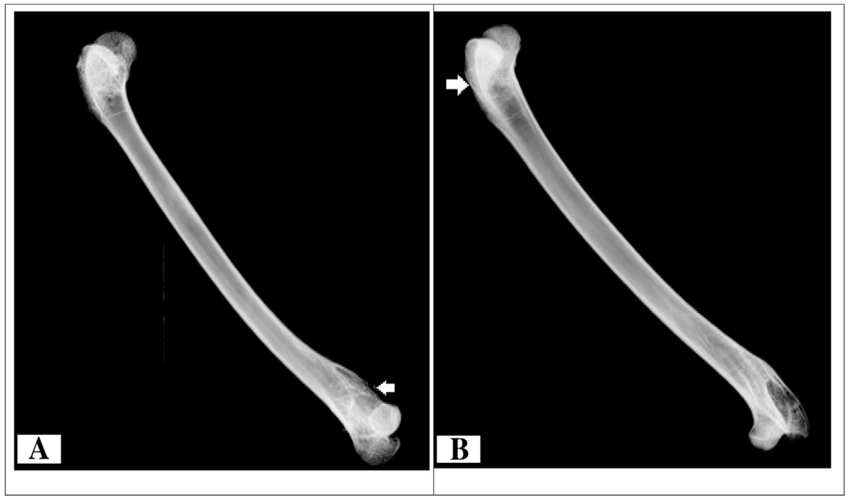


Fig. 3. A: Femur of snowshoe hare from the reference site showing osteoporosis and avulsion cortical fracture with observation of bone fragments of the lower third of the femur (avulsion fracture is defined as separation of a small fragment of bone cortex at the site of attachment of a ligament or tendon). 3B: cortical fracture along the neck of the femur, including disruption of the normal cortical architecture.

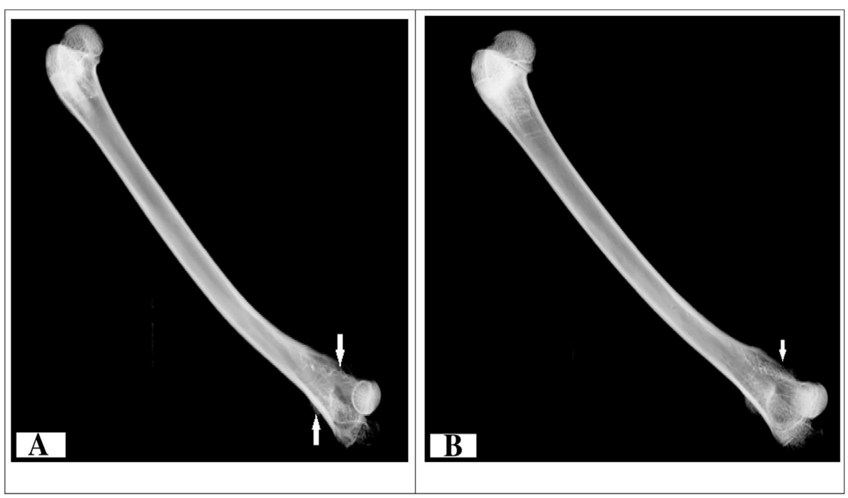


Fig. 4. A: Massive disruption/fragmentations and fractures of the cortices on both sides of the lower part of the femur were noted to be associated with osteolysis of the femoral condyle and massive bone loss. 4B: Osteoporosis with bone loss over the lower third of the femur, including disruptive mechanism of the cortices was noted.

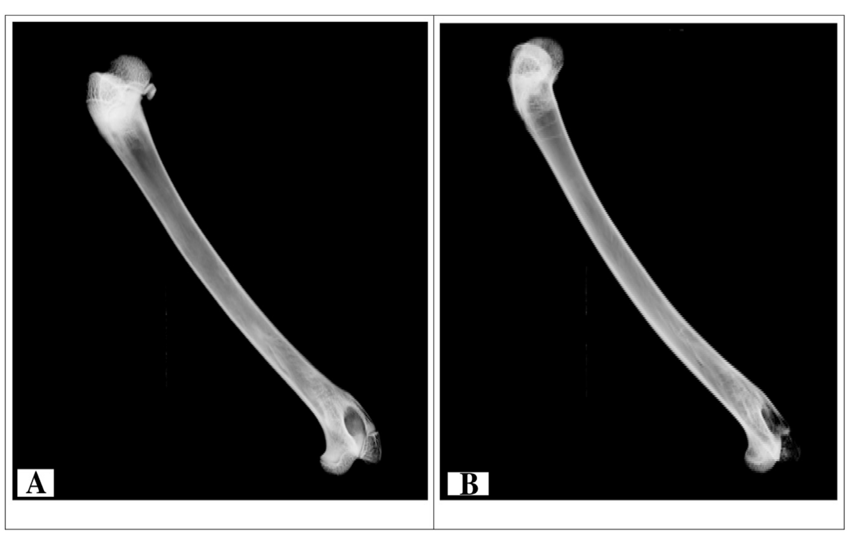


Fig. 5. A: Osteoporosis was noticeably accompanied with dysplastic (small) capital femoral epiphysis, with the upper 1/3rd of the diaphysis showing osteoporotic changes, whereas the lower third showed no bone loss. Further, the cortices of the mid-diaphysis were noticeably thin and liable to fracture. 5B: osteoporosis, and irregular lines of ossification of the lower third of the femur.

or tendon) with observation of bone fragments of the lower third of the femur (Fig. 3A). Traces of minute cortical fractures along the neck of the femur were noted to be accompanied with disruption of the normal cortical architecture, and minute avulsion fracture (Fig. 3B). Massive disruption/fragmentations and fractures of the cortices on both sides of the femur were noted to be associated with osteolysis of the femoral condyle and massive bone loss (Fig. 4A). Several specimens examined exhibited osteopenia with bone loss over the lower third of the femur, including disruptive mechanism of the cortices (Fig. 4B). Osteoporosis was noticeably accompanied with dysplastic (small) capital femoral epiphysis, with the upper 1/3rd of the diaphysis showing osteoporotic changes, whereas the lower third showed no bone loss. Further, the cortices of the mid-diaphysis were noticeably thin, which may make them liable to fracture (Fig. 5A). Other bone specimens exhibited osteopenia, and irregular lines of ossification of the lower third of the femur (Fig. 5B). The vertebrae samples examined showed thinner and dysplatic transverse as well as spinous processes (Fig. 6B and D). It is important to clarify here that As was below detection limit in all the bones from the reference site, but Cd generally accumulated in the bones at levels that was similar to the specimens from the Giant mine area. As a result, it is likely that the manifestation of osteoporosis and other bone abnormalities observed in the reference animals may be due to chronic Cd exposure at levels similar to the animals from the mine site. Our preliminary data in our Part 1 paper already indicate that the levels of Cd in the nails and bones of hares from the reference area were not statistically different from the mine site specimens ($p > 0.05$).

4.5. Case area

In the mine area, bone of hares showed evidence of trabecular sclerosis over the epi-metaphyseal regions of the femoral head. A scan of the lower 2/3rd of the femur showed small islands of well-demarcated sclerosis seen at the diaphyseal area extending downwards to involve the epi-metaphyseal areas as well (white arrow near femoral neck of Fig. 7A). Furthermore, cortical hyperostosis was also observed involving the periosteum (white arrow along the shaft) and endosteal hyperostosis (black arrow). It seems that arsenic inhibited the osteoclastic remodelling. Periosteal hyperostosis was also noted to be associated with nodule-like formations. The femoral neck of the hares showed osteoporotic changes, whereas the metaphysis shows longitudinally sclerosed trabeculae (top white arrow on Fig. 7B). Tiny spots of sclerosis were noted to be more marked at mid-diaphysis and extended downwards. The inferior femur showed signs of demineralization and profound osteoporosis (white arrow in the inferior femur on Fig. 7B). The bone specimens of other hares exhibited small focal islands of sclerosis, spreading along the whole shaft. Osteoporotic changes were also observed along the capital femoral epiphysis shaft and the inferior femur. Dysplastic capital femoral epiphysis, osteoporosis, and thin cortices with progressive osteopenia were commonly observed in specimens from the area (Fig. 8A and B). The vertebrae showed evidence of severe osteoporosis of the transverse process with small islands of well-demarcated sclerosis noted along the transverse processes. It is likely that these small islands represent arsenic replacing phosphorus in the bone matrix (Fig. 9A and B). In addition, the vertebrae also exhibited islands of well-demarcated sclerosis and cyst-like changes on top of osteoporosis invading the outer and the inner cortices of the transverse processes and parts of the vertebral body.

Visual inspection of representative bone samples from both locations further confirmed that there was a difference in the surface texture of the bones, with the specimen from the reference site showing a typical smooth surface of normal phenotype (Fig. 10A), whereas that from the mine area had diffuse dissemination of small islands of pathological ossification along the periosteum (Fig. 10B). A previous animal study conducted by Odstrcil et al. (2010) showed that exposure to low levels of arsenic increases the thickness of the growth plate cartilage, as well as inhibit endochronal ossification. While dysplasia of the proximal capital femoral epiphysis was noted in the femur from the mine area, the bone of the reference animal showed normal growth including proximal capital femoral epiphysis. To the best of our knowledge, the result presented in this paper is the first attempt to describe the pattern of bone abnormalities in wild terrestrial small mammals inhabiting arsenic endemic areas, and exposed to environmental levels of Cd. Although hares from both location showed evidence of chronic As and Cd exposure, only Cd tended to accumulate consistently in the bones of the animals, and, it is likely that the deterioration of bone quality as noted in the radiographic assessment may have been induced mainly by the influence of Cd and less of As. The constellation of skeletal abnormalities noted such as osteoporosis, cortical fractures, sclerosis, and cyst like changes in the femurs and vertebrae of hares from both locations suggests that the developing bone is a target for metal and metalloid toxicity, and that concomitant environmental exposures to As and Cd may be associated with varying severities of osteological abnormalities, as observed in our study.

5. Conclusion

Chronic exposure to As and Cd has been suggested to play a role in the etiology of various bone disorders, including osteoporosis, and the present study is among the first documentation of bone abnormalties in wild terrestrial small mammals inhabiting As contaminated areas

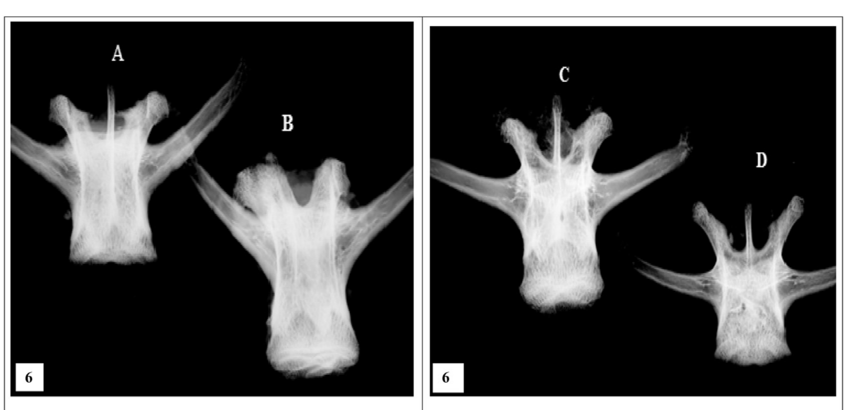


Fig. 6. The vertebrae samples examined showed smaller vertebral body including thinner and dysplatic transverse as well as spinous processes associated with irregular lines of abnormal ossification (Fig. 6B and D).

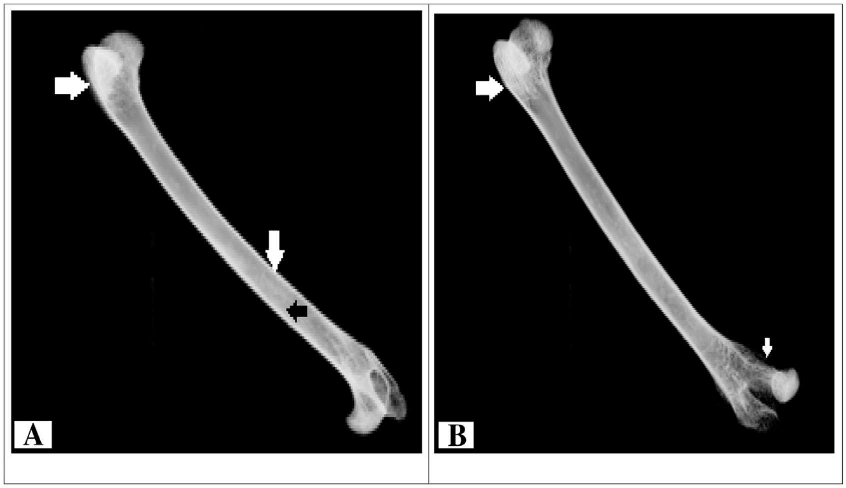


Fig. 7. A: Abnormal sclerosis over metaphyseal regions of the femoral head. Lower 2/3rd of the femur of hare showed small islands and irregular lines of ossification. 7B: The femoral neck of the hares showed osteoporotic changes. The inferior femur showed signs of osteoporosis.

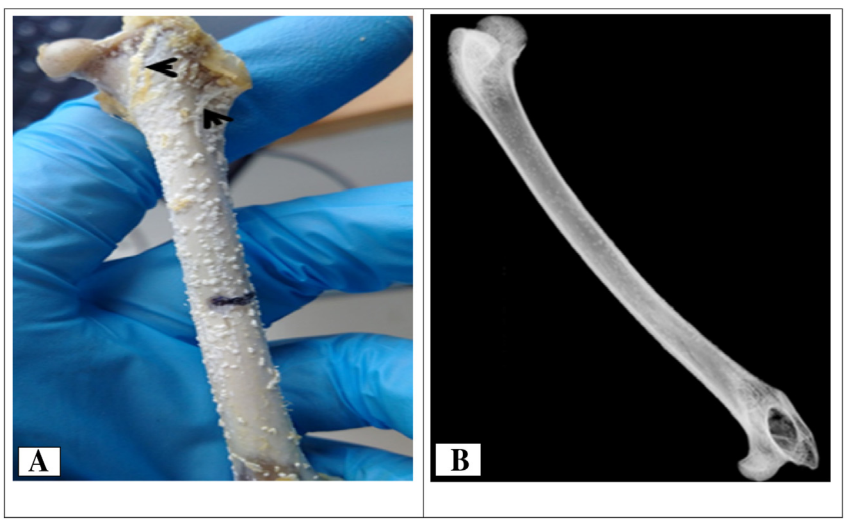


Fig. 8. A: Small focal islands of sclerotic spots spreading over the periosteum (periosteum is an extremely thin region of non-calcified tissue on the exterior of bones). 8B: Osteoporotic changes were also observed along the capital femoral epiphysis shaft and the inferior femur.

due to the influence of mining activities and geogenic sources. The results from this present study provide baseline information regarding the effects of As and Cd exposure on bone quality of snowshoe hares inhabiting the Yellowknife area. The results of our investigation show that combined chronic exposure to environmental levels of As and Cd induces bone abnormalities, including bone fragility and increases the risk of osteoporosis in exposed snowshoe hares breeding in the study area. Despite the differences in As and Cd exposure in snowshoe hares from the study areas, the bone pathology of hares from both locations were generally noted to be similar. Both groups generally exhibited osteoporosis and cortical fractures of the femurs, whereas the vertebrae of hares from the mine area show reduced BA and TA. It is also likely that chronic exposure to elevated levels of As and Cd from geogenic sources may have also contributed to the development of pathological changes in the bones of the case and reference animals. As a result, caution should be taken when interpreting the results of this study to fully consider the role of natural geology as a potential risk factor for increased exposure to contaminants, and which may also play a role in inducing developmental effects, including bone disorders. The results of our investigation confirm that bones may constitute a highly sensitive indicator for assessing the combined effects of chronic arsenicosis and Cd

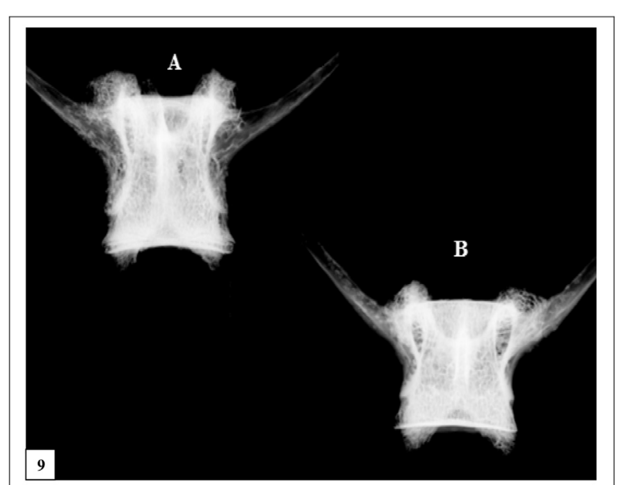


Fig. 9. (A and B): severe osteoporosis of the transverse processes with small sclerotic spots noted along the transverse processes.

Fig. 10. Comparing the phenotype of two femura of two different animals. Dysplasia of the proximal capital femoral epiphysis (10B- from Giant Mine area). Bone (10A- from Reference location) shows normal proximal capital femoral epiphysis. Diffuse dissemination of small islands of pathological ossification along the perisoteum (10B). Bone (10A) shows normal phenotype.

exposure in wildlife population breeding in the Yellowknife area, and other contaminated environment.

Acknowledgements

The authors would like to acknowledge the financial support (Research Fellowship/Research Assistant Award) received from the Aurora Research Institute to conduct this study. We also appreciate the technical support provided by Prof. David Goltzman and Dr. Yongjun Xiao from the Centre for Bone and Periodontal Research, McGill University.

References

Akbal, A., Yılmaz, H., Tutkun, E., 2014. Arsenic exposure associated with decreased bone mineralization in male. The Aging Male 17 (4), 256–258.
Bromstad, M.J., Wrye, L.A., Jamieson, H.E., 2017. The characterization, mobility, and persistence of roaster-derived arsenic in soils at Giant Mine, NWT. Appl. Geochem. 82, 102–118.
Brzóska, M.M., Moniuszko-Jakoniuk, J., 2004. Low-level exposure to cadmium during the lifetime increases the risk of osteoporosis and fractures of the lumbar spine in the elderly: studies on a rat model of human environmental exposure. Toxicol. Sci. 82 (2), 468–477.
Brzóska, M.M., Moniuszko-Jakoniuk, J., 2005. Effect of chronic exposure to cadmium on the mineral status and mechanical properties of lumbar spine of male rats. Toxicol. Lett. 157 (2), 161–172.
Eisler, R., 1994. A Review of Arsenic Hazards to Plants and Animals with Emphasis on Fishery and Wildlife Resources.
Environmental Sciences Group, 2001. Arsenic Levels in the Yellowknife Area: Distinguishing Btewen Natural and Anthropogenic Inputs. http://www.reviewboard.ca/upload/project_document/EA0809-001_Document_on_Arsenic_Levels_in_Yellowknife_-_Exhibit_1.PDF
Florencio-Silva, R., Sasso, G.R.D.S., Sasso-Cerri, E., Simões, M.J., Cerri, P.S., 2015. Biology of bone tissue: structure, function, and factors that influence bone cells. BioMed Res. Int.
Government of the Northwest Territories, 2016. A Guide to Mineral Deposits. http://www.iti.gov.nt.ca/sites/www.iti.gov.nt.ca/files/mineraldeposites2-nocrops.pdf.
Hocking, D., Kuchar, P., Plambeck, J.A., Smith, R.A., 1978. The impact of gold smelter emissions on vegetation and soils of a sub-arctic forest-tundra transition ecosystem. J. Air Pollut. Control. Assoc. 28 (2), 133–137.
Hu, Y.C., Cheng, H.L., Hsieh, B.S., Huang, L.W., Huang, T.C., Chang, K.L., 2012. Arsenic trioxide affects bone remodeling by effects on osteoblast differentiation and function. Bone 50 (6), 1406–1415.
Kido, S., 2013. Secondary osteoporosis or secondary contributors to bone loss in fracture. Bone metabolism and heavy metals (cadmium and iron). Clin. Calcium 23 (9), 1299–1306.
Koch, I., Wang, L., Ollson, C.A., Cullen, W.R., Reimer, K.J., 2000. The predominance of inorganic arsenic species in plants from Yellowknife, Northwest Territories, Canada. Environ. Sci. Technol. 34 (1), 22–26.
Koch, I., Mace, J.V., Reimer, K.J., 2005. Arsenic speciation in terrestrial birds from Yellowknife, Northwest Territories, Canada: the unexpected finding of arsenobetaine. Environ. Toxicol. Chem. 24 (6), 1468–1474.
Lim, H.S., Lee, H.H., Kim, T.H., Lee, B.R., 2016. Relationship between heavy metal exposure and bone mineral density in Korean adult. J. Bone Metab. 23 (4), 223–231.
Odstrcil, A.D.C.A., Carino, S.N., Ricci, J.C.D., Mandalunis, P.M., 2010. Effect of arsenic in endochondral ossification of experimental animals. Exp. Toxicol. Pathol. 62 (3), 243–249.
Saunders, J.R., Hough, C., Knopper, L.D., Koch, I., Reimer, K.J., 2011. Arsenic transformations in terrestrial small mammal food chains from contaminated sites in Canada. J. Environ. Monit. 13 (6), 1784–1792.
Turner, C.H., 2002. Biomechanics of bone: determinants of skeletal fragility and bone quality. Osteoporos. Int. 13 (2), 97–104.
Turner, C.H., 2006. Bone strength: current concepts. Ann. N. Y. Acad. Sci. 1068 (1), 429–446.