Prion, 9:48–58, 2015
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ISSN: 1933-6896 print / 1933-690X online
DOI: 10.1080/19336896.2015.1019194

Metals in obex and retropharyngeal lymph nodes of Illinois white-tailed deer and their variations associated with CWD status

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ABSTRACT. Prion proteins (PrP^C) are cell membrane glycoproteins that can be found in many cell types, but specially in neurons. Many studies have suggested PrP^C's participation in metal transport and cellular protection against stress in the central nervous system (CNS). On the other hand PrP^{Sc}, the misfolded isoform of PrP^C and the pathogenic agent in transmissible spongiform encephalopathies (TSE), has been associated with brain metal dyshomeostasis in prion diseases. Thus, changes in metal concentration associated with protein misfolding and aggregation have been reported for human and animal prion diseases, as well as for other neurodegenerative disorders, such as Parkinson's and Alzheimer's disease. The use of metal concentrations in tissues as surrogate markers for early detection of TSEs has been suggested. Studies on the accumulation of metals in free-ranging white-tailed deer have not been conducted. This study established concentrations of copper, iron, manganese, and magnesium in 2 diagnostic tissues used for CWD testing (obex and retropharyngeal lymph nodes (RLN)). We compared these concentrations between tissues and in relation to CWD status. We established reference intervals (RIs) for these metals and explored their ability to discriminate between CWD-positive and CWD-negative animals. Our results indicate that independent of CWD status, white-tailed deer accumulate higher concentrations of Fe, Mn and Mg in

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Received November 21, 2014; Revised January 25, 2015; Accepted February 9, 2015.
Color versions of one or more figures in this article can be found online at www.tandfonline.com/kprn.

RLN than in obex. White-tailed deer infected with CWD accumulated significantly lower concentrations of Mn and Fe than CWD-negative deer. These patterns differed from other species infected with prion diseases. Overlapping values between CWD positive and negative groups indicate that evaluation of these metals in obex and RLN may not be appropriate as a diagnostic tool for CWD infection in white-tailed deer. Because the CWD-negative deer were included in constructing the RIs, high specificities were expected and should be interpreted with caution. Due to the low sensitivity derived from the RIs, we do not recommend using metal concentrations for disease discrimination.

KEYWORDS. chronic wasting disease, copper, prion, iron, metals imbalance, manganese, magnesium, transmissible spongiform encephalopathy

ABBREVIATIONS. AAS, atomic absorption spectroscopy; AD, Alzheimer disease; ASVCP, American Society for Veterinary Clinical Pathology; BBB, blood brain barrier; CI, confidence intervals; CNS, central nervous system; CP, choroid plexus; Cu, copper; CWD, chronic wasting disease; Fe, iron; ICP-MS, inductively coupled plasma mass spectrometry; IDNR, Illinois Department of Natural Resources; ISTC, Illinois Sustainable Technology Center; ISWS, Illinois State Water Survey; Mg, magnesium; Mn, manganese; PD, Parkinson disease; PRNP, prion protein gene; PrP^{C}, cellular prion protein; PrP^{Sc}, abnormal isoform of prion protein; RIs, reference intervals; RLN, retropharyngeal lymph nodes; SOD, superoxide dismutase; SSURGO, Soil Survey Geographic database; STATSGO, State Soil Geographic Database; Tf, transferrin; TfR, transferrin receptors; TSE, transmissible spongiform enchephalopathies

INTRODUCTION

Chronic wasting disease (CWD) is a prion disease of cervids that was first detected in Illinois white-tailed deer in 2002.[1,2] Prion diseases (or transmissible spongiform enchephalopathies, TSE) are characterized by neural accumulation of the misfolded isoform (PrP^{Sc}) of the normal cell-surface glycoprotein, the prion protein (PrP^{C}).[3,4] Obex and retropharyngeal lymph nodes (RLN) are important tissues for CWD diagnosis, as PrP^{Sc} is detectable in both tissues even at sub-clinical stages of CWD.[5]

PrP^{C} can be found throughout the body with higher concentrations at presynaptic membranes in the central nervous system (CNS). The exact physiological roles of PrP^{C} are still under investigation, but many studies have suggested its participation in the metabolism of metals and its role as antioxidant (superoxide dismutase (SOD)-like activity).[6–10] Although the potential redox activity of PrP^{C} has been previously demonstrated, this is controversial since other studies dispute the SOD function of PrP^{C}.[11,12] The interaction of PrP^{C} with metals such as iron (Fe), copper (Cu) and manganese (Mn) is important, since these metals serve as cofactors for enzymes, and play important roles in mammalian biochemical processes. Physiological requirements of dietary metals differ with species, sex and age.[13,14] In deer, dietary metals uptake can change with habitat, season and home range.[15] Due to the role of these metals in neurological processes, their influx into the CNS is tightly regulated via the blood brain barrier (BBB) and the choroid plexus (CP) leading ultimately to differences in their concentrations in the CNS vs. other tissues.[16]

PrP^{Sc}, on the other hand, is the pathogenic agent in all TSEs, as changes in conformation of PrP^{C} to PrP^{Sc} induce pathological modifications such as: neurotoxicity due to disruption of PrP^{C} normal function and imbalance in metal homeostasis in the brain, physical damage of membranes by PrP^{Sc} aggregation, and apoptosis as a result of intracellular PrP^{Sc} accumulation.[10,17-19] Moreover, TSE neurotoxicity, associated with imbalance in brain homeostasis and deregulation of metals, has been previously observed for other neurodegenerative disorders such as Parkinson's disease (PD) and Alzheimer's disease (AD).[9]

PrP^{C}-metal interactions differ with each metal and may play a physiological or pathological role. For example, binding of PrP^{C} to Cu is linked to PrP^{C} stability and antioxidant activity

at brain synapses, protecting the cells from oxidative damage and neurodegeneration.[8,20,21] On the other hand, Mn has been implicated in facilitating the conversion of PrP^{C} to PrP^{Sc}, favoring PrP^{Sc} aggregation and resistance to protease degradation.[19,22] Moreover, the loss of PrP^{C} functions may increase intracellular free radicals and oxidative damage.[9,18]

TSEs like Scrapie and Sporadic Creutzfeldt-Jacob disease have been linked to changes in Fe metabolism that contribute to accumulation of PrP^{Sc}, sequestration of Fe in PrP^{Sc}-ferritin complexes, and neurotoxicity associated to upregulation of Fe intake into the brain.[10,17] Furthermore, Fe binding to PrP^{C} contributes to conformational changes that provide PrP^{Sc} stability and resistance to proteinase K (PK) digestion. The formation of PrP^{Sc}-ferritin complexes resulting from the upregulation of PrP^{C} and ferritin within the lysosome, ultimately contributes to PrP^{Sc} generation and propagation, even in absence of the infectious PrP^{Sc}.[17] Additionally, increased total and redox-active Fe (II), together with increased levels of transport and storage proteins, such as transferrin (Tf) and transferrin receptors (TfR), play a crucial role in Fe dyshomestasis and associated neurotoxicity in prion infected human, mouse and hamster brains.[10] Interestingly, reduced levels of Fe in presence of excess total Fe was found to be a characteristic associated with prion diseases, but not with other neurodegenerative diseases such as AD and PD.[10]

A combined decrease in Cu with an increase in Mn in blood and brain has been detected prior to disease onset in BSE infected cattle and Scrapie-infected mice and sheep, as well as in brain synaptosomes of Scrapie-infected mice.[18,23,24] Similar changes in liver have been reported in chronic wasting disease (CWD) infected free-ranging mule deer; while low levels of magnesium (Mg) have been reported in the brain of CWD-infected elk.[25,26] Metal analyses of blood and tissue have been suggested as surrogate markers for prion infection.[18,23,25]

Besides the analysis of TSE neurotoxicity associated with imbalance in brain homeostasis and deregulation of metals, many studies have considered the role of environmental factors, such as soil properties, on TSE transmission. Soil characteristics such as pH, organic matter, clay content and soil metals can influence TSE transmission, as these soil properties can impact protein stability, prion persistence in the environment and TSE infectivity.[27-30] However, other soil characteristics, such as natural oxidants like manganese oxides (MnO_2), appear to interfere with the conversion of PrP^{C} to PrP^{Sc}.[31]

To our knowledge there is no data regarding the concentration of Mn, Cu, Fe and Mg in obex and RLN of white-tailed deer even though these tissues are the preferred sites for diagnostic sampling and testing for CWD, and target sites for PrP^{Sc} accumulation during infection. The objectives of this study were: 1) to compare concentrations of Cu, Fe, Mn and Mg between tissues (obex and RLN) and CWD status, 2) to establish reference intervals (RIs) for Cu, Mn, Fe and Mg in obex and RLN of Illinois free-ranging white-tailed deer; and 3) to explore the utility of the reference intervals established in objective 2 for discriminating between CWD-positive and CWD-negative animals. We hypothesize that concentrations of metals will be different between tissues and CWD status, and that the reference intervals for metal concentrations established in the study can be used to discriminate CWD-positive and CWD-negative animals.

RESULTS

Descriptive statistics for metal concentrations by tissues and CWD status are summarized in **Table 1** and **Table 2**. Due to right-skewness of the distributions of the metal concentrations, logarithmic (base 10) transformation was applied and geometric means and geometric standard deviations were calculated. At this level, medians were consistently higher in RLN than in obex regardless of CWD status (**Table 1**) for all metals except for Cu in negative samples.

Results of the mixed models showed that Cu and Mg:Mn were the only measures that did not differ significantly between tissues, while Cu and Mg were not significantly different between CWD-negative and CWD-positive deer (**Table 2**). Soil pH was included as a

TABLE 1. Summary of metal concentrations in retropharyngeal lymph nodes (RLN) and obex from chronic wasting disease (CWD) negative and positive free-ranging white-tailed deer (*Odocoileus virginianus*)

		Negative			Positive		
Metal	Tissue	GMean (GSD)	Median (IQR)	Range	GMean (GSD)	Median (IQR)	Range
Cu	RLN	4.37 (2.0)	4.5 (2.7 – 5.9)	1.1 – 141.0	6.03 (2.19)	5.5 (3.9 – 8.5)	1.6 – 36.2
	Obex	4.79 (1.66)	4.9 (3.4 – 6.6)	1.2 – 17.3	5.13 (2.00)	5.3 (3.1 – 6.8)	1.8 – 57.8
Fe	RLN	851.14 (2.95)	634.4 (350.4 – 1638.7)	125.9 – 16325.0	407.38 (1.86)	425.84 (278.1 – 626.9)	90.6 – 1348.7
	Obex	190.55 (2.14)	174.8 (110.3 – 283.6)	53.6 – 1826.7	117.49 (1.74)	114.5 (82.4 – 136.9)	50.2 – 652.2
Mg	RLN	354.81 (1.82)	375.3 (258.4 – 486.5)	63.3 – 1928.4	501.19 (1.78)	459.8 (383.5 – 778.6)	101.1 – 2196.4
	Obex	97.72 (1.95)	94.2 (64.5 – 141.6)	22.9 – 563.1	141.25 (1.35)	134.2 (120.9 – 158.5)	91.0 – 367.4
Mn	RLN	10.0 (4.37)	12.0 (3.0 – 33.2)	0.4 – 173.5	2.24 (1.95)	2.2 (1.5 – 3.5)	0.5 – 16.9
	Obex	3.24 (3.55)	4.4 (1.4 – 8.3)	0.2 – 53.6	0.66 (1.91)	0.5 (0.5 – 0.8)	0.3 – 6.6

Values are μg/g dry weight. Geometric mean (GMean) and Geometric standard deviation (GSD). Interquartile range (IQR) shows the 25th percentile to the 75th percentile range. The number of samples include Negatives RLN (83), Obex (82); Positives RLN (31), Obex (34).

covariate in final models of Fe, Mn, Mg and metal ratios, and age, clay content and organic matter were added as covariates in models of Mg concentrations. Age was the only covariate included in models of Cu concentration. The estimated reference intervals for metals and ratios in RLN and obex are summarized in **Table 3**. Poor sensitivity for reference intervals on discriminating CWD status was found for all metals and ratios in both tissues (**Table 3**).

DISCUSSION

Metal concentrations differed by CWD status, with significantly reduced Mn and Fe in CWD-positive compared to CWD-negative white-tailed deer. Our results differ from previous findings where mice and ruminants with neurodegenerative disorders like CWD, scrapie and BSE showed lower levels of Mg and Cu, higher levels of Mn, and no significant changes in Fe.[18,23,25,26] This suggests that results among species infected with TSEs should not be extrapolated.

Prion protein expression and disease-associated neurotoxicity has been related to disruption of the metabolism of Cu and Fe dyshomeostasis at early stages of prion infection and before disease onset[6,18,23,32] It is not known if differences between CWD negative and positive deer in the current study are a

TABLE 2. Results of the mixed models for comparing concentrations of Cu, Fe, Mn and Mg and the Cu to Mn and Mg to Mn ratios between tissues (retropharyngeal lymph nodes(RLN) and obex) and between chronic wasting disease (CWD) negative and positive free-ranging white-tailed deer (*Odocoileus virginianus*) in Illinois

Metal	RLN vs. Obex Coefficient (SE) p-value	CWD+ vs. CWD- Coefficient (SE) p-value	Covariates included
Cu	−0.010 (0.035) 0.773	0.078 (0.043) 0.071	age
Fe	0.620 (0.028) <0.01	−0.158 (0.062) 0.013	soil pH
Mg	0.561 (0.023) <0.01	0.076 (0.043) 0.078	age, soil pH, clay content and organic matter
Mn	0.501 (0.051) <0.01	−0.554 (0.087) <0.01	soil pH
Cu:Mn	−0.512 (0.056) <0.01	0.653 (0.089) <0.01	soil pH
Mg:Mn	0.058 (0.039) 0.141	0.658 (0.098) <0.01	soil pH

Deer nested within county (i.e., deer(county)) was included as the random effect in the mixed models. A backward selection procedure was applied to select significant ($p < 0.05$) covariates, including sex and age of deer and soil properties (pH, clay content and organic matter) to be included in the final models. Log_{10} transformation was applied to metal concentrations (dependent variables).

TABLE 3. Reference intervals derived from CWD-negative deer. Sensitivity and specificity of discriminating CWD status using the reference intervals (RIs)

Metal	Tissue (n)	Lower limit (90% CI)	Upper limit (90% CI)	Sensitivity (95% CI)	Specificity (95% CI)
Cu	RLN (83)	1.4 (1.2–1.7)	22.9 (15.9–32.3)	0.17 (0.07 – 0.34)	0.96 (0.90 – 0.99)
	Obex (82)	1.7 (1.5 – 2.1)	13.0 (11.05 – 15.3)	0.11 (0.03 – 0.27)	0.94 (0.87 – 0.98)
Fe	RLN (83)	162.5 (136.2 – 191.4)	NA	0.20 (0.08 – 0.37)	0.99 (0.93 – 1)
	Obex (82)	58.5 (51.8 – 66.9)	NA	0.06 (0.01 – 0.19)	0.96 (0.90 – 0.99)
Mg	RLN (83)	112.5 (90.4 – 142.2)	1192.0 (970.8 – 1466.0)	0.17 (0.07 – 0.34)	0.93 (0.85 – 0.97)
	Obex (82)	24.5 (19.7 – 31.0)	359.7 (287.4 – 445.0)	0.06 (0.01 – 0.19)	0.93 (0.85 – 0.97)
Mn	RLN (83)	0.5 (0.3 – 0.9)	210.0 (146.1 – 355.5)	0.11 (0.03 – 0.27)	0.99 (0.93 – 1)
	Obex (82)	0.3 (0.2 – 0.4)	47.6 (33.7 – 69.1)	0.03 (0 – 0.15)	0.96 (0.90 – 0.99)
Cu:Mn	RLN (83)	0.04 (0.03 – 0.05)	31.6 (11.4 – 137.1)	0.11 (0.03 – 0.27)	0.99 (0.93 – 1)
	Obex (82)	0.1 (0.1 – 0.2)	20.9 (12.9 – 32.6)	0.03 (0 – 0.15)	0.98 (0.92 – 1)
Mg:Mn	RLN (83)	1.6 (1.1 – 2.4)	1216.5 (687.4 – 1939.2)	0.11 (0.03 – 0.27)	1 (0.96 – 1)
	Obex (82)	2.7 (2.1 – 3.5)	1127.0 (435.6 – 2504.5)	0.03 (0 – 0.15)	0.98 (0.92 – 1)

Values are μg/g dry weight. RLN = retropharyngeal lymph nodes; CI = Confidence intervals; NA: due to small n. Upper and lower limits of the RIs were used to classify each deer into 2 groups, negative (deer within the upper and lower limits of the RIs) or positive (deer outside the upper and lower limits of the RIs) group. Sensitivity and specificity were computed by comparing the CWD status of each deer against the groups (negative or positive) estimated using the RIs.

driving factor in disease pathogenesis or a consequence of neuropathological changes. We did not know when animals acquire infection or if they had developed clinical signs at the time of sample collection. However, tolerance to dietary metals between species, breeds and physiology may explain these differences, and could affect incubation period and prion disease onset.[33]

Other factors such as age, genotype, type of tissue, geographic location and home range could influence metal concentrations, and may explain differences between infected and non-infected animals, and between species.[23,28,30,34,35] For instance, Rocky mountain elk (*Cervus elaphus nelsoni*) with 2 copies of methionine at PrP codon 132 (132MM) accumulated higher levels of Mg in the brain than elk with leucine at codon 132. In contrast, PRNP genotype did not influence blood levels of Cu, Fe, Mn or Mg in sheep,[23] indicating that the effects of genotype are species-specific. Certain PRNP polymorphisms are correlated with CWD resistance in white-tailed deer,[34] and it would be interesting to determine if this association is related to differences in metal accumulation among genotypes. Regardless, differences in the accumulation of each metal need to be considered for each genotype, species and type of tissue. Moreover, we should be cautious when interpreting changes in concentrations between and within species, and when extrapolating results from one tissue to another.

Our results suggest that both mineral accumulation and fluctuation in tissue concentration of Illinois white-tailed deer covary with pH characteristics of Illinois soil (**Table 2**). Recent research in Illinois suggested that there was a spatial correlation of CWD risk with alkaline soils, such as those found in the northern part of the state where CWD clusters concentrate.[35] In addition, there was evidence of an association between CWD risk and a lower percentage of clay and a higher percentage of sand in soils. The analysis by Ruiz et al.[35] was carried out at a spatial scale based on geographic units of one square mile, thus making direct comparison difficult with the current study carried out at the level of the county. The collection of field samples and more precise measures of soil ingestion by deer in this region would help to better elucidate the relationship between metals, soils and CWD.

Several studies analyzed the effect of formalin-fixation and storage time (weeks, months and years) on the concentration of metals in stored biological tissues.[36,37] However, we did not detect a time dependent decrease in the concentrations of metals from the samples used in this study. The highest

and lower concentrations of each metal were found in both, early and late samples, suggesting that differences in concentrations of metals in our samples were not time dependent or associated with leaking into formalin (unpublished observations). While evaluation of leaking of metals from formaldehyde fixed tissues was beyond the scope of this study, we recognize that leaking could have occurred. A study comparing the differences in metal concentration across time from both fixed and frozen tissues would be the best tool to establish leakage of metals into formalin from formaldehyde fixed tissues.

Differences between tissues were detected for all metals except for Cu, with higher levels of Fe, Mn and Mg in RLN than in obex. Differences of metal accumulation between tissues may be due to variations between uptake mechanisms across the blood brain barrier (BBB) and the choroid plexus (CP). Disturbance of the uptake mechanism and transport proteins may occur at different stages of the disease progression for different metals.[18,23,38] For example, changes in Fe are usually accompanied by changes in Mn because similar proteins transport both metals. Although, differences in metal accumulation between obex and RLN could be expected, higher accumulation of metal in RLN of CWD-negative deer deserves further attention since CWD has been suggested to affect RLN before it affect the CNS, and Mn and Fe could act as potential risk factors for prion protein expression and conversion, and subsequent neurotoxicity.[5,6,19,22]

CWD-negative white-tailed deer showed broad ranges of metal accumulation in both tissues (**Table 1**). Similarly, broad ranges have been detected in obex of free ranging mule deer, and in blood, brain, liver and muscle of cattle not infected with TSEs.[23,25] However, CWD-negative white-tailed deer had slightly higher geometric mean concentrations of Cu (**Table 1**) than reported in the brains of cattle and sheep (4.6 and 4.3 μg/g, respectively), but lower than in the brain of elk (11.85 μg/g dry-weight), and mule deer (10.3 μg/g dry-weight). Differences in mean concentrations across species extended to Fe, Mn and Mg, corroborating the importance of defining reference intervals for different species.[23,25,26]

This study is the first to estimate reference intervals for concentration of metals in obex and RLN of a free-ranging population of white-tailed deer. We recognize that our limited sample size prevented us from reaching the desired precision, and increasing sample size in future studies will help to determine more reliable reference intervals. Nonetheless, we have followed the American Society for Veterinary Clinical Pathology (ASVCP) guidelines for establishing reference intervals from small sample sizes that will benefit the veterinary medical community, as this could be used as a reference for future studies of metals in white-tailed deer.[39] The RIs established in this study were further explored for their utility in discriminating between CWD positive and negative deer. Because the CWD-negative deer were included in constructing the RIs, high specificities were expected and should be interpreted with caution. With the observed low sensitivities, we do not recommend using RIs for disease discrimination and further investigation is needed.

We know that different tissues accumulate different concentrations of metals, and differences in the accumulation of metals have been associated with TSE pathology. However, while CWD was associated with changes in concentrations of metals in obex and RLN in white-tailed deer, their role in PrP regulation, homeostasis, and neurotoxicity is still poorly understood. Metal concentrations should be further analyzed in multiple tissues that can be easily collected from live animals to facilitate proper interpretations of variations across tissues within an individual. Other important aspects should be also considered, as dietary needs and changes in metal concentration may be related to habitat composition and genotype. A variety of ecological and physiological factors associated with metal metabolism in white-tailed deer need to be understood before we can effectively use concentrations of metals in tissues as surrogate markers for prion infection.

MATERIAL AND METHODS

Sample Collection

Between 2002 and 2009 the Illinois Department of Natural Resources (IDNR), CWD surveillance/management program, sampled more than 43000 white-tailed deer (http://www.dnr.illinois.gov/programs/CWD/Documents/CWDAnnualReport20092010.pdf; last accessed 25 January 2015). The location (county name) of harvested deer was provided to IDNR biologists by hunters. Age was estimated by IDNR biologists on the basis of tooth morphology.[40] Obex and RLN samples were preserved in metal free polypropylene containers prefilled with 10% neutral buffered formalin. All samples collected between 2002 and 2009 were tested at the Illinois Department of Agriculture's Animal Disease Laboratories using immunohistochemistry staining — the gold standard diagnostic test suggested by the USDA for CWD detection. Tissues were classified by the laboratories as either "positive" or "non-detected" (thereafter referred to as negative) for CWD. A randomly selected subset of these formaldehyde fixed samples was stored at the University of Illinois after determination of CWD status.

The samples included in this study were obtained from the available subset of stored samples (n = 5000) at the university of Illinois. We selected a total of 116 obex and 114 RLN from 128 adult deer 2-4 years old (83 CWD-negative and 35 CWD-positive white-tailed deer).

Selection Criteria of Negative CWD Samples

Selection criteria for CWD-negative samples was based on age (adult deer between 2 to 4 years old), existing tissue (as long as both obex and RLN were available for each individual), and distribution around the state of Illinois based on counties where deer were harvested (a similar number of females and males representing 14 counties from north and 14 counties from south Illinois). A total of 83 deer (43 females, 40 males) with negative CWD test results were randomly chosen from available samples that met the selection criteria. Concentrations of Cu, Fe, Mn and Mg were evaluated in 82 obex and all 83 of the RLN. The missing obex was lost in shipment after the sample was digested and sent for ICP-MS analysis.

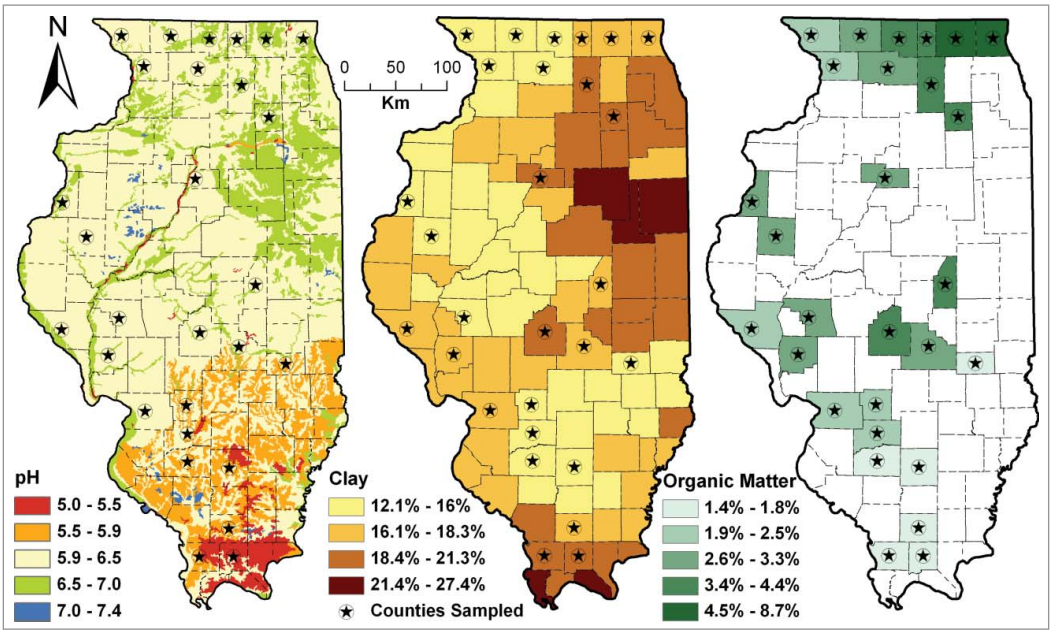
Selection Criteria of Positive CWD Samples

CWD-positive deer in Illinois were only found in 5 counties in the northern part of the state, from which the positive samples were selected (**Fig. 1**). Due to limited CWD-positive samples available, we included all the samples that met the age criteria, even if we did not have both tissues (RLN and obex) for that animal. A total of 35 deer (18 females, 17 males) with positive CWD test results were prepared for metal analysis. Concentrations of Cu, Fe, Mn and Mg were limited to 34 obex and 31 RLN of the 35 deer.

Sample Digestion and Metal Analysis

We used a modified tissue digestion procedure from the Association of Official Analytical Chemists (Procedure 9.1.01 – AOAC, 1995).[41] Obex and RLN (0.2 – 0.7g) were placed in Pyrex tubes, washed twice with 50 ml of deionized water to remove excess formaldehyde and left in 50 ml of deionized water for an overnight wash. Following the overnight wash, water was decanted and wet weight was determined. Samples were then dried for 24 hours at 109°C and dry weight obtained. A mixture of chloroform-methanol-water at ratios of 2:1:0.5 respectively was added to each sample for overnight lipid extraction. Samples were reweighed and digested with 5ml of concentrated nitric acid (Trace Metal grade) for 48 hours. If undigested tissue remained after 48 hours, samples were placed in a water bath (40°C) for 2 to 6 hours until the digestion was complete

FIGURE 1. Counties sampled and soil properties. Maps of the state of Illinois showing the 28 counties where samples for metal analysis were collected from the CWD management and surveillance program (2002–2009). Soil maps indicate the distribution of average pH (left), percentage of clay (middle), and percentage of organic matter (right). Soils measures were developed from the US Department of Agriculture county level (SSURGO) and state level (STATSGO) data, depending on the availability of the soil characteristic of interest.



and the sample was clear and suitable for analysis. Following digestion, deionized water was added to dilute the sample to a 20% volume/volume concentration.

Digested samples were evaluated at the Illinois Sustainable Technology Center (ISTC) and the Illinois State Water Survey (ISWS), University of Illinois at Urbana-Champaign. Levels of Cu, Mg and Mn were determined by inductively coupled plasma mass spectrometry (ICP-MS), using scandium and yttrium as internal standards. Iron levels were obtained using atomic absorption spectroscopy (AAS). Analytical duplicates and spikes were included as quality control measures. Ratios for Cu:Mn and Mg:Mn were calculated from these results.

Environmental Variables

Soil survey data from both the more detailed SSURGO program and less detailed state level STATSGO program were obtained from data created by the US Department of Agriculture, depending on the availability of the soil characteristic of interest in each program (http://www.nrcs.usda.gov/wps/portal/nrcs/site/soils/home/). Average pH, percentage organic matter and percentage clay were the variables estimated for each county and included in our environmental models. For the pH and clay variables, we used raster data summaries of STATSGO data available in the 1 km resolution GeoSTAC spatial dataset (http://geostac.tamu.edu/summary). For each county, the mean pH and percentage of clay was estimated using an area-weighted mean from the raster data. The organic matter variable – a measure of the amount of plant and animal residue in the soil at various stages of decomposition – was available only in the more detailed data and was estimated for each county from 28 different soil maps. Then, the county organic matter variable was calculated as an area-weighted mean from the original soil polygons.

Statistical Analysis

Descriptive statistics were computed with MedCalc (Version 12.7.0, MedCalc Software, Ostend, Belgium) to describe the distributions of metal concentrations by type of tissue and CWD status. Mixed models were used to compare concentrations of metals between tissues and CWD status (equation 1). Deer nested within county was included as the random effect in the models. A backward selection procedure was performed to select significant ($p < 0.05$) covariates to be included in the final models. SAS for Window (version 9.2, SAS Inc., Cary, NC) was used for data analysis. A p value of < 0.05 was considered significant.

$$Y_{ij} = \beta_{0j} + \beta_{1j}X_{1ij} + \beta_{2j}X_{2ij} + \underline{\beta_j X_{ij}} + e_{ij} \quad (1)$$

where metal concentrations and ratios are the dependent variables (Y_{ij}; each in the separated model) at individual deer (i) and county level (j), while tissues (X_{1ij}; obex and RLN) and CWD status (X_{2ij}) were the deer-level independent variables. Sex, age of deer, and soil properties (pH, clay content and organic matter) were investigated as covariates ($\underline{\beta_j X_{ij}}$). e_{ij} is the error term.

CWD-negative deer were used to estimate reference intervals for the metal concentrations. Box-Cox transformations were applied to CWD-negative samples and the robust method for small sample size was used to derive reference intervals (RIs) and 90% confidence intervals (CI).[42] The limits of these RIs were used as the cutoff values to classify each deer into positive (if the measure was outside the RI) or negative (if the measure was inside the RI) group. The dichotomous results were compared against the CWD status of deer to compute sensitivities and specificities. 95% binomial confidence intervals (CI) for sensitivity and specificity were also presented.

DISCLOSURE OF POTENTIAL CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

ACKNOWLEDGMENTS

The authors thank the Illinois Department of Natural Resources for collecting samples, sharing data and for their continued effort in the management of CWD. The authors also thank the hunting community in Illinois for their assistance and willingness to have their deer tested for CWD. In addition we want to thank R.V. Morales for reviewing this manuscript, S. Fredebaugh for laboratory assistance, and W. M. Brown for GIS support.

FUNDING

This research was funded by the US Fish & Wildlife Service Federal Aid in Wildlife Restoration Project (W-146-R) and the University of Illinois Office of the Vice Chancellor for Research, with additional support by the University of Illinois, Illinois Natural History Survey.

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