

MULTIPLE METALS EXPOSURE AND NEUROTOXIC RISK IN BALD EAGLES
(*HALIAEETUS LEUCOCEPHALUS*) FROM TWO GREAT LAKES STATES

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(Submitted 18 August 2011; Returned for Revision 2 October 2011; Accepted 25 October 2011)

Abstract—In the present study, the authors determined concentrations of several elements (As, Cd, Co, Cu, Cr, Mn, Pb, Sb, Zn) in the brains and livers of 46 bald eagles (*Haliaeetus leucocephalus*) from two Great Lakes states, Michigan and Minnesota. To explore whether exposures are of neurological concern, the authors assessed their associations with neurochemical receptors (*N*-methyl-D-aspartate [NMDA] and γ -aminobutyric acid A [GABA(A)]) and enzymes (glutamine synthetase [GS] and glutamic acid decarboxylase [GAD]) that play critical roles in vertebrate neurobehavior and reproduction. For most elements, levels in the livers and brains did not differ between region and gender. Hepatic Pb levels averaged 33.1 ppm (dry wt), 30.4% of all carcasses exceeded proposed avian Pb thresholds (>26.4 ppm), and in 30.8% of the birds examined evidence of Pb pellets or fragments was found. Significant changes in the activities of GS and GAD were related to brain concentrations of several metals (Pb, Cd, Co, Cu, Zn). No relationships were found among any of the nine elements and NMDA or GABA(A) receptor levels. When combined with the authors' previous study on these same eagles that showed Hg-associated alterations in GS, GAD, and NMDA receptor levels, the present research suggests that bald eagles are exposed to various elements, especially Pb and Hg, that are capable of causing changes in GABAergic and glutamatergic neurotransmission. The functional significance of these neurochemical changes warrants attention. *Environ. Toxicol. Chem.* 2012;31:623–631. © 2011 SETAC

Keywords—Neurochemical biomarkers Neurotoxicology Wildlife toxicology Heavy metals Brain Lead

INTRODUCTION

Bald eagles (*Haliaeetus leucocephalus*) are apex predators that bioaccumulate many environmental contaminants [1,2]. Studies on bald eagles across North America have documented appreciable levels of heavy metals, particularly Hg, in their tissues [3–6]. Although Hg exposure and its potential neurological impacts in bald eagles have been recently documented [7,8], much less is known about the exposure of eagles to other metals and potential neurological effects.

Animal neurobehavior and reproduction are influenced by brain neurochemistry, particularly by the neurotransmitters glutamate and γ -aminobutyric acid (GABA) [9,10]. Toxicant-induced changes in the function of neurochemical receptors and enzymes may result in subclinical effects that precede overt damage. Several wildlife studies have documented Hg-associated changes to neurochemical receptors and enzymes [7,8,11–17]. In addition to Hg, rodent studies have shown that several transition metals, such as Cd, Ag, Co, Cu, Pb, Mn, and Zn, also impair neurochemical systems [18–20]. However, in the field of wildlife toxicology, the neurochemical effects of these other metals have been poorly studied.

Two recent studies on bald eagles showed significant associations between brain Hg levels and neurochemical receptors (*N*-methyl-D-aspartic acid [NMDA] receptor and muscarinic acetylcholine receptor) and enzymes (acetyl cholinesterase, glutamic acid decarboxylase [GAD], and glutamine synthetase [GS]) [7,8], suggesting that Hg may be of neurological concern to this species. Although previous studies have shown significant tissue burdens (for example, in liver, kidney, and feathers

[5,21]) of other toxic and essential elements, few have explored levels in the brain and whether they may be of neurotoxicological concern. The present study was undertaken to determine concentrations of several elements (Cr, Mn, Co, Cu, Zn, As, Cd, Sb, Pb) in brain and liver tissues of 46 wild bald eagles collected from two U.S. states in the Great Lakes region, Michigan and Minnesota. Furthermore, these elements were compared to neurochemical enzymes (GAD, GS) and receptors (NMDA, GABA) previously characterized by Rutkiewicz et al. [8] in these same tissues.

MATERIALS AND METHODS

Animals

Bald eagles used for the present study were part of another study concerning Hg exposure and neurochemical effects [8]. In the present study, we investigated a subset of carcasses ($n = 46$) that were selected from Michigan and Minnesota and matched according to available biological data (e.g., age, gender). Carcasses were provided by the Michigan Department of Natural Resources and Environment, The Raptor Center (University of Minnesota), and Minnesota Department of Natural Resources. From necropsy reports, birds died of various causes in the field (mainly trauma) or during rehabilitation. Carcasses were collected between 2002 and 2010 and stored at -20°C until tissue collection. All necropsied birds were adults as determined by plumage. Of birds for which sex data were available, 12 were male and 18 were female. Extracted tissues were stored at -80°C until all analyses. Proper U.S. Fish and Wildlife Service permits were obtained for this work.

Elemental analysis

Based on methods the authors published previously [22], the following elements were analyzed in whole brain and liver

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Published online 14 December 2011 in Wiley Online Library
(wileyonlinelibrary.com).

tissue: Cr, Mn, Co, Cu, Zn, As, Cd, Sb, and Pb. Subsamples of liver and brain tissues were dried for 72 h at 60°C, and then 0.1 to 0.3 g of the dried sample was digested with 1 ml concentrated nitric acid (70% nitric acid; Optima grade, Fisher Scientific) overnight at room temperature. The following day, each digest was heated for 1 h at 60°C, and then for 3 to 5 h at 90°C. The final digest was diluted with Milli-Q water to 2% nitric acid.

All elements were detected using an inductively coupled plasma mass spectrometer (Agilent 7500c, Agilent Technologies) equipped with a quadrupole analyzer and octopole collision/reaction cell pressurized with either a H₂ or He reaction gas. Sample uptake was 0.4 ml/min from a peristaltic pump with 1.2 L/min Ar carrier gas through a Babbington-style nebulizer into a Peltier-cooled double-pass spray chamber at 2°C; 1.0 L/min auxiliary Ar and 12.0 L/min plasma gas Ar were added for a total of 14.2 L/min separated from nickel cones by a sampling depth of 8.5 mm. The inductively coupled plasma mass spectrometer was tuned under standard settings by running the manufacturer's recommended tuning solution of 10 ppb of Li, Y, Ce, Tl, and Co (Agilent internal standard mix) for resolution and sensitivity. Interference levels were reduced by optimizing plasma conditions to produce low oxide and doubly charged ions (formation ratio of <1.0%) and residual matrix interferences were removed using the collision/reaction processes in the Octopole Reaction System.

A series of rigorous analytical quality control measures were used. All biological samples were handled in a Class 100 and 1000 clean lab. Glassware, plasticware, and Teflon[®]-coated tubes were acid-washed (cleaned, soaked in 10% nitric acid for 24 h) prior to use. Accuracy and precision were measured using the Canadian National Research Council's DOLT-4. In addition, each batch run contained procedural blanks and replicate runs. Samples for which a contaminant was detected but with a concentration below the analytical detection limit were assigned a value of one-half the detection limit. For each particular element, the analytical detection limit was calculated as the concentration of the element that gave a detectable signal above the background noise at greater than the 99% confidence level, so that the detection limit was calculated as the mean of blanks plus three times the standard deviation of the mean. Detection limits were as follows: Cr (0.09 µg/L), Mn (0.17 µg/L), Co (0.05 µg/L), Cu (0.06 µg/L), Zn (0.48 µg/L), As (0.01 µg/L), Cd (0.05 µg/L), Sb (0.01 µg/L), and Pb (0.01 µg/L). Accuracy (closeness to actual value) ranged from 92.4 to 116%. The reproducibility (precision) of each element was within 6% of expected values.

Neurochemical assays

Cellular homogenates, enzyme supernatants, and membranes were prepared according to modifications of a procedure described by Rutkiewicz et al. [8] and Nam et al. [23]. Briefly, subsamples of brain (cerebrum) were homogenized in ice-cold Tris-buffer (50 mM NaH₂PO₄, 5 mM KCl, 120 mM NaCl, pH 7.4), and the supernatant was collected following a 15-min centrifugation at 15,000 g (4°C). Cellular membranes were then isolated by centrifuging the supernatant at 48,000 g for 15 min (4°C), washing three times, and resuspending in buffer. The concentration of protein in the supernatant preparation was determined with the Bradford assay using bovine serum albumin as the standard. Prepared samples were stored at -80°C until analysis.

Neurochemical enzyme activity for GS and GAD were measured according to procedures and technical equipment detailed by Rutkiewicz et al. [8]. For GS activity, assays were

performed on supernatant (400 µg protein/ml; incubation at 37°C) and absorbance (500 nm) was measured to determine γ -glutamyl hydroxymate concentration and specific activity. For GAD (as total) activity, the reaction mixture containing 100 µg of protein was used. The reaction was started with the addition of 10 mM glutamate containing 0.3 µCi [¹⁴C]-glutamic acid (260 mCi/mmol; PerkinElmer) (45 min incubation at 37°C) and terminated with injection of 0.5 ml of 0.25 M HCl (60 min incubation at 37°C). After soaking in a scintillation cocktail (PerkinElmer), radioactivity was measured on a liquid scintillation counter (Wallac 1450 Microbeta Plus, PerkinElmer). Samples were assayed in triplicate and duplicate for GS and GAD, respectively. Both within and between-day variability averaged less than 20% for both assays. Enzyme activities were expressed as nanomoles/milligram/minute (GS activity) or femtomoles/milligram/hour (GAD activity).

Receptor binding assays were performed using cellular membranes according to previously published methods [8,23]. For both receptors, protein (30 µg) was suspended in a 100 µl buffer (NMDA: 50 mM Tris buffer [pH 7.4] containing 100 µM glycine and glutamate; GABA: 50 mM Tris buffer [pH 7.4]). Samples were incubated at room temperature with 5 nM [³H]MK-801 (27.5 Ci/mmol) for 2 h (for NMDA) or on ice with 2 nM [³H]flunitrazepam (81.4 Ci/mmol) for 30 min (for GABA). Non-specific binding was determined through coinubation with 100 µM unlabeled MK-801 (NMDA) or 20 µM clonazepam (GABA). Samples were assayed in quadruplicate. Variability, which averaged less than 20%, was monitored through internal, pooled controls. Binding is reported as femtomoles of radioisotope bound per milligram of membrane protein.

Statistical analyses

Data were analyzed using PASW Statistics (Ver 17.0). All concentrations are reported as mean \pm standard deviation on a dry weight basis unless otherwise noted. Preliminary analysis included descriptive statistics for all measures. Normality and homoscedasticity of data were tested by using the Kolmogorov-Smirnov test and the Levene's test (F_{\max} test), respectively. All tissue element concentrations were log-transformed prior to statistical analyses (one-way analysis of variance or *t* tests). Pearson correlations were used to determine relationships between concentrations of tissue elements, as well as associations with neurochemical biomarkers. Quartile analyses were established using the mean (\pm standard error) and the 25% and 75% interquartile Pb cutoffs, and were then used to compare the differences among quartiles of Pb measures following one-way analysis of variance. Associations among elements were analyzed using factor (principal component) analysis. A *p* value of <0.05 was considered significant in all analyses.

RESULTS

Nine different elements were analyzed in the liver and brain tissue of 46 bald eagles (Table 1). The mean moisture content in the liver and brain was 66.1 \pm 5.8% and 79.7 \pm 3.4%, respectively. In general, mean metal concentrations were higher in the liver than the brain. When stratified across sampling regions (Minnesota vs Michigan, and two regions within both states) and gender, no significant differences were detected in the liver or brain, except for brain Mn and brain Zn, which exhibited regional differences (Table 1).

Correlative approaches were used to explore associations between concentrations of elements between the liver and the brain (Fig. 1), as well as among the elements in the liver (Table 2)

Table 1. Concentrations of elements (mean ± standard deviation; µg/g dry wt) in brain and liver from adult bald eagle (*Haliaeetus leucocephalus*) carcasses

	As	Cd	Co	Cr	Cu	Mn ^a	Pb	Sb	Zn ^a	
Brain	All animals	0.007 ± 0.014	0.027 ± 0.014	0.038 ± 0.012	1.47 ± 0.30	24.6 ± 9.6	3.88 ± 4.99	0.008 ± 0.023	56.8 ± 9.5	
	Location									
	MI LP (n = 13)	0.005 ± 0.008	0.030 ± 0.011	0.035 ± 0.010	1.30 ± 0.24	22.9 ± 10.2	1.57 ± 0.29 A	3.16 ± 4.18	0.006 ± 0.006	49.7 ± 6.9 C
	MI UP (n = 13)	0.011 ± 0.020	0.037 ± 0.015	0.038 ± 0.010	1.68 ± 0.27	25.6 ± 8.0	1.74 ± 0.22 AB	4.88 ± 5.67	0.015 ± 0.044	57.4 ± 6.9 CD
	MI NW (n = 11)	0.009 ± 0.015	0.021 ± 0.013	0.039 ± 0.010	1.43 ± 0.24	31.1 ± 9.3	2.08 ± 0.34 B	5.23 ± 6.27	0.003 ± 0.002	63.5 ± 7.9 D
	MI EC (n = 9)	0.003 ± 0.006	0.018 ± 0.005	0.041 ± 0.020	1.47 ± 0.33	17.5 ± 6.2	2.05 ± 0.57 B	1.80 ± 2.56	0.008 ± 0.004	58.0 ± 11.6 CD
	Gender									
	Male (n = 12)	0.011 ± 0.021	0.032 ± 0.014	0.036 ± 0.011	1.51 ± 0.42	27.9 ± 13.4	1.80 ± 0.43	4.64 ± 6.37	0.016 ± 0.046	57.7 ± 12.3
	Female (n = 18)	0.003 ± 0.009	0.030 ± 0.014	0.036 ± 0.012	1.44 ± 0.25	23.5 ± 9.9	1.79 ± 0.50	2.53 ± 3.15	0.005 ± 0.004	55.6 ± 10.0
	Unknown (n = 16)	0.005 ± 0.011	0.009 ± 0.012	0.022 ± 0.012	1.23 ± 0.25	13.4 ± 4.6	1.60 ± 0.26	2.77 ± 5.48	0.001 ± 0.006	46.5 ± 6.4
Liver	Pb shot ^b	0.006 ± 0.007	0.035 ± 0.015	0.036 ± 0.010	1.51 ± 0.34	24.1 ± 9.6	1.67 ± 0.23	2.07 ± 3.92	0.003 ± 0.003	52.9 ± 8.7
	All animals	0.014 ± 0.026	0.031 ± 0.008	0.037 ± 0.010	1.45 ± 0.28	24.5 ± 8.4	1.61 ± 0.34	6.82 ± 6.13	0.026 ± 0.055	55.0 ± 5.5
	Location									
	MI LP (n = 13)	0.013 ± 0.035	0.357 ± 0.236	0.080 ± 0.037	1.28 ± 0.18	32.3 ± 25.3	14.5 ± 7.5	33.1 ± 57.7	0.225 ± 0.756	237 ± 186
	MI UP (n = 13)	0.006 ± 0.014	0.366 ± 0.127	0.080 ± 0.038	1.40 ± 0.09	31.2 ± 21.5	13.9 ± 6.4	13.2 ± 22.1	0.106 ± 0.259	198 ± 185
	MI NW (n = 11)	0.002 ± 0.005	0.481 ± 0.309	0.073 ± 0.021	1.31 ± 0.19	30.4 ± 21.2	12.8 ± 4.4	36.5 ± 68.4	0.046 ± 0.059	225 ± 143
	MI EC (n = 9)	0.009 ± 0.021	0.312 ± 0.252	0.092 ± 0.045	1.17 ± 0.13	47.1 ± 35.8	17.4 ± 10.5	68.6 ± 77.5	0.712 ± 1.460	301 ± 214
	Gender									
	Male (n = 12)	0.015 ± 0.030	0.220 ± 0.133	0.073 ± 0.045	1.21 ± 0.23	18.3 ± 10.9	14.0 ± 8.4	13.8 ± 25.2	0.063 ± 0.133	230 ± 215
	Female (n = 18)	0.002 ± 0.007	0.434 ± 0.206	0.079 ± 0.031	1.32 ± 0.12	38.7 ± 23.3	14.3 ± 6.2	24.0 ± 42.4	0.087 ± 0.175	229 ± 186
Pb shot ^b	Unknown (n = 16)	0.021 ± 0.048	0.380 ± 0.291	0.088 ± 0.041	1.33 ± 0.16	16.5 ± 9.2	15.9 ± 27.0	0.099 ± 0.232	276 ± 195	
	Negative (n = 18)	0.012 ± 0.031	0.273 ± 0.156	0.070 ± 0.036	1.20 ± 0.22	37.0 ± 33.9	12.3 ± 5.6	0.472 ± 1.237	198 ± 172	
	Positive (n = 8)	0.006 ± 0.012	0.447 ± 0.269	0.084 ± 0.031	1.34 ± 0.17	29.4 ± 18.6	13.3 ± 5.9	21.2 ± 52.4	0.082 ± 0.220	217 ± 184
		0.003 ± 0.003	0.370 ± 0.153	0.061 ± 0.024	1.40 ± 0.07	34.0 ± 26.5	13.6 ± 4.5	33.1 ± 50.8	0.062 ± 0.078	200 ± 111

^a Letters denotes significant differences ($p < 0.05$) for brain Mn and Zn between locations.

^b Fluoroscopic analysis was used only in the birds ($n = 26$) from Michigan.

MI = Michigan; MN = Minnesota; LP = Lower Peninsula; UP = Upper Peninsula; NW = Northwest; EC = East Central.

and brain (Table 3). Lead, in particular, correlated with Cd, Sb, and Zn in the liver (Table 2), and with Cd, Cr, Sb, and Se in the brain (Table 3). Many of the other elements correlated with each other in both tissues (Tables 2 and 3). Significant correlations among established toxic elements (As, Cd, and Pb) were observed between the liver and brain (Fig. 1).

Based on proposed threshold values from the literature [24–26], an opportunity was present here to put our hepatic tissue residue findings (As, Cd, Cu, Hg, Mn, Pb, Zn) into a broader context (Fig. 2). Residues of essential (e.g., Cu, Mn, Zn) and toxic (e.g., As, Cd) hepatic elements were lower than proposed threshold values and were generally within ranges found in healthy animals including birds [24,25]. In contrast, hepatic levels of Pb averaged 33.1 ppm (range: 0.20–215 ppm), and 14 of 46 eagles (30.4% of the birds; average hepatic lead concentration: 104 ppm; range: 42.2–215 ppm) exceeded levels suggestive of Pb poisoning (26.4 ppm dry wt, assuming 70% moisture content) in birds of prey [26]. Liver Hg concentrations in our previous results [8] exceeded the toxic threshold (16.7 ppm; [24]) in 26.1% of birds. When the Pb and Hg data are considered together, 10.8% of birds had hepatic levels of both toxic metals that exceeded suggested toxic thresholds.

Data were subjected to principal component analysis to determine if hepatic elements clustered in groups (Fig. 3). The first eigenvalue estimated from the matrix accounted for 28.6% of the total variance, and two eigenvalues explained 45.4% of the total variance. A diagnostic assessment of the scatterplots indicated two groups, the first consisting of Pb, Sb, and As, and the other comprising all other elements, including Hg.

We previously analyzed a suite of neurochemical biomarkers in these same eagles in relation to Hg exposure [8], and the present study provided an opportunity to explore associations with other elements. There were no gender-associated differences in neurochemical biomarkers. Correlative approaches were used to assess the associations between log-transformed concentrations of elements in the brain and four neurochemical biomarkers (Table 4). Brain Cu and Zn were positively correlated with GS activity (Table 4). The activity of brain GAD was negatively correlated with Co, Cd, and Pb. Multivariate analysis of the overall model indicated that GAD activity was most strongly related to Pb, followed by Cd, inorganic Hg, and others (including methylmercury, total Hg) ($df = 11, F = 330.18, p < 0.05$). Specific binding to the NMDA and GABA(A) (benzodiazepine) receptors did not correlate significantly with all elements investigated (Table 4).

Because of the high Pb exposures reported in the present study and the innate neurotoxic potential of Pb across vertebrates, the eagles were stratified into quartiles according to brain Pb levels. From this analysis, a significant decrease in GAD activity ($df = 3, F = 3.1, p < 0.05$) was found between the first (range: <0.75 ppm Pb) and fourth (range: 6.4–18.3 ppm Pb) quartile groups ($n = 10$ or 11 per group) (Fig. 4). No significant changes were observed between Pb levels and other neurochemical biomarkers (Fig. 4).

Fluoroscopic analysis was used only in the birds from Michigan ($n = 26$), and eight individuals had Pb pellets or fragments (range: 1–19 pieces) in their digestive tracts. Although levels of tissue Pb in birds having confirmed Pb pellet exposures were higher than the other birds, this increase was not to a level of statistical significance (Table 1). Figure 5 shows associations of neurochemical biomarkers in relation to birds exposed to Pb shot. There were no changes of neurochemical biomarkers between birds that were negative and birds that were positive for Pb shot.

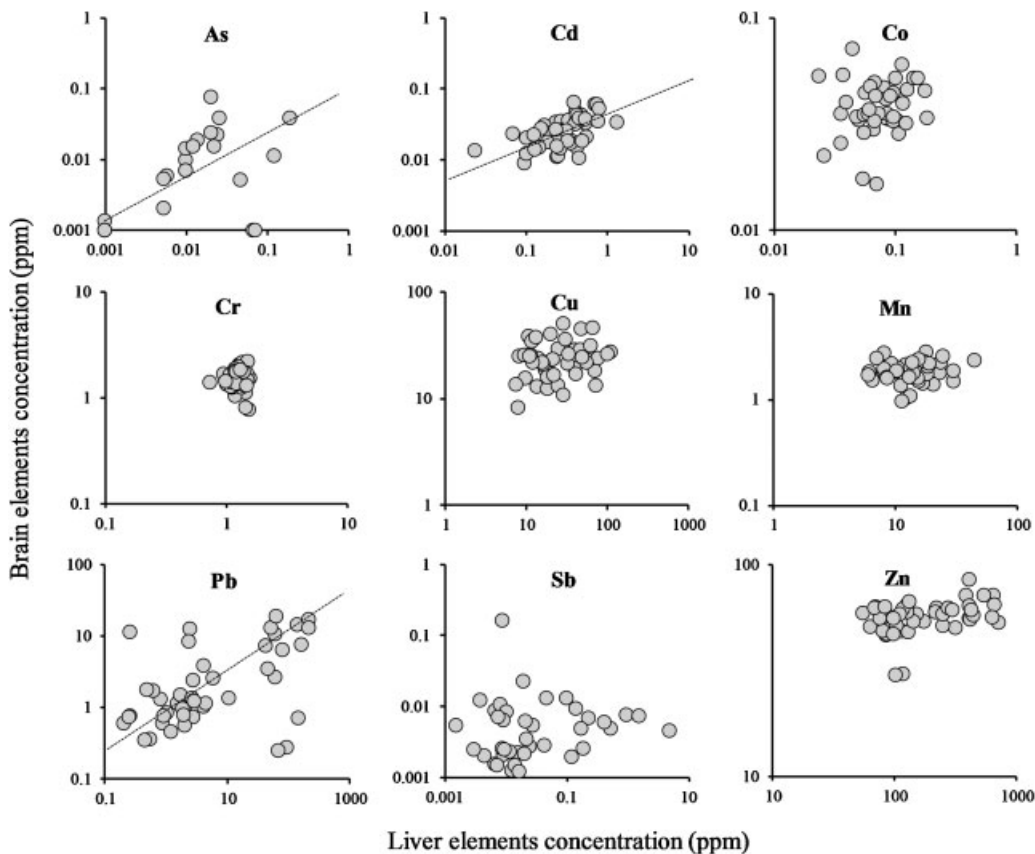


Fig. 1. Correlation of elements between liver and brain in bald eagles (*Haliaeetus leucocephalus*). Statistically significant correlations were found for As ($r=0.377$, $p < 0.05$), Cd ($r=0.544$, $p < 0.001$), and Pb ($r=0.583$, $p < 0.001$).

DISCUSSION

To our knowledge, this is the first report outlining concentrations of several elements, including heavy metals of ecologic and neurotoxic concern, in the brain tissue of bald eagles. The major findings were that 30.4% of all carcasses investigated had Pb residues associated with intoxication (>26.4 ppm dry wt) and that significant changes of physiologically important neurochemical enzymes (GS, GAD) were related to brain concentrations of Pb, Cd, Co, Cu, and Zn.

The threat of Pb intoxication has been well documented in raptors, including bald eagles. This concern largely arises from the ingestion of Pb shot projectiles or fragments embedded in their prey. In our study, concentrations of hepatic Pb (mean:

33.1 ppm; range: 0.20–215 ppm) are comparable to values suggestive of Pb poisoning in some raptorial species [26], including the bald eagle [27,28], golden eagle (*Aquila chrysaetos*) [28], and white-tailed eagle (*Haliaeetus albicilla*) [29]. A conservative liver threshold for toxic effects in some birds of prey, especially Falconiformes, is proposed to be 26.4 ppm [26]. In the present study, 30.4% of eagles investigated exceeded this value. Lead is an established neurotoxicant, and severe Pb intoxication can cause neuropathological damage in the brain (encephalopathy) manifested by an impairment of the blood-brain barrier [30]. Brain Pb was significantly correlated with hepatic Pb levels (Fig. 1). The resulting regression equation ($Y = 1.142x^{0.5721}$, where Y = brain Hg and x = liver Hg) enabled us to predict a brain threshold for toxicity of approximately

Table 2. Correlation matrix among hepatic elements^a

	As	Cd	Co	Cr	Cu	Hg	Mn	Pb	Sb	Se
Cd	0.313*	–	–	–	–	–	–	–	–	–
Co	–0.091	0.292*	–	–	–	–	–	–	–	–
Cr	0.021	0.080	0.128	–	–	–	–	–	–	–
Cu	–0.096	0.364*	0.390**	0.037	–	–	–	–	–	–
Hg	–0.127	0.405*	0.261	0.164	0.367*	–	–	–	–	–
Mn	0.250	0.358*	0.287	0.120	0.138	0.227	–	–	–	–
Pb	0.006	0.300*	0.104	–0.121	0.273	0.171	0.263	–	–	–
Sb	0.027	0.192	0.026	–0.089	0.161	–0.066	0.007	0.742***	–	–
Se	–0.062	0.310*	0.229	0.264	0.502**	0.760***	0.153	0.053	–0.017	–
Zn	–0.055	0.438**	0.443**	–0.001	0.305*	0.408**	0.724***	0.458**	0.132	0.227

^aMercury (Hg) and Se data were obtained from the study of Rutkiewicz et al. [8].

* $p < 0.05$;

** $p < 0.01$;

*** $p < 0.001$.

Table 3. Correlation matrix among brain elements^a

	As	Cd	Co	Cr	Cu	Hg	Mn	Pb	Sb	Se
Cd	0.090	–	–	–	–	–	–	–	–	–
Co	–0.132	0.164	–	–	–	–	–	–	–	–
Cr	0.027	0.387**	0.265	–	–	–	–	–	–	–
Cu	0.224	0.118	0.130	0.094	–	–	–	–	–	–
Hg	0.164	0.376*	–0.019	0.078	0.218	–	–	–	–	–
Mn	–0.105	–0.210	0.341*	0.452**	0.210	–0.154	–	–	–	–
Pb	–0.018	0.360*	0.198	0.313*	0.050	0.046	0.063	–	–	–
Sb	0.002	0.011	0.136	0.269	–0.238	0.029	0.194	0.327*	–	–
Se	0.407**	0.499**	0.187	0.278	0.447**	0.597***	0.053	0.303*	–0.030	–
Zn	0.010	–0.106	0.472**	0.290	0.323*	–0.076	0.591***	0.237	0.069	0.194

^aMercury (Hg) and Se data were obtained from the study of Rutkiewicz et al. [8].

* $p < 0.05$;
 ** $p < 0.01$;
 *** $p < 0.001$.

6.4 ppm (associated with 26.4 ppm in liver), which was exceeded by 23.9% of eagles in this study. These results suggest that tissue Pb residues are of toxicological or neurotoxicological concern in a sizeable number of bald eagles across the Great Lakes region (Michigan and Minnesota).

Legislative action has imposed restrictions on the use of Pb shot for hunting in several regions, but because of the modern and historical ubiquity of Pb shot use and the persistence of Pb in the environment, Pb exposure is still of concern to wildlife across North America. The U.S. Environmental Protection Agency (U.S. EPA) estimated that in 2005 approximately 80,000 tons of Pb shot entered the U.S. environment from shooting ranges (http://www.epa.gov/Region2/waste/leadshot/epa_mp.pdf). Our findings, via fluoroscopy or confirmed observations, demonstrated that 30.8% of adult carcasses collected from Michigan had Pb pellets or fragments in their digestive tracts. Though birds with confirmed Pb exposures (as Pb shot

presence) had higher levels in their tissues, similar hepatic and brain Pb residues were found between the two groups (Table 1). This lack of statistical difference may be related to a low sample size, extensive Pb exposure to general eagle populations regardless of Pb uptake pathway (e.g., direct consumption of Pb fragments through their prey or secondary uptake of Pb from Pb-contaminated prey), as well as some uncertainty regarding the residency time and subsequent kinetics of Pb tissue distribution of the observed/fluoroscoped Pb shot in each animal.

The metallic formulation of Pb shots vary, but they generally comprise Pb (>90%), with some Sb (1–5%), and other elements (As, Cu, Ni, and Zn) [31]. Antimony (Sb) is used primarily as a hardener for Pb ammunition. Although several wildlife toxicology studies have characterized Pb exposure, fewer have explored Sb levels. In our study, concentrations of Sb in livers and brains averaged 0.225 ppm (range: 0.005–4.89 ppm) and 0.008 ppm (range: 0.001–0.161 ppm), respectively (Table 1). Antimony concentrations were significantly associated with Pb levels in both liver and brain (Tables 2 and 3), thus suggesting coexposures to Pb and Sb. Although Sb and its compounds are environmental pollutants suspected of being genotoxic and carcinogenic [32,33], very few studies have reported on Sb

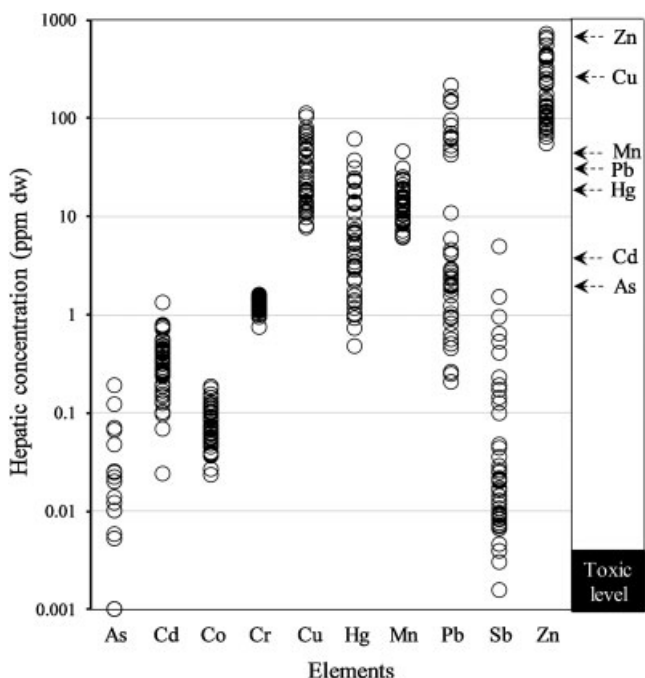


Fig. 2. Hepatic concentrations of elements (Hg data from Rutkiewicz et al. [8]) in bald eagles (*Haliaeetus leucocephalus*) in relation to risk threshold values proposed by Zillioux et al. [24], Puls [25], and Beyer et al. [26].

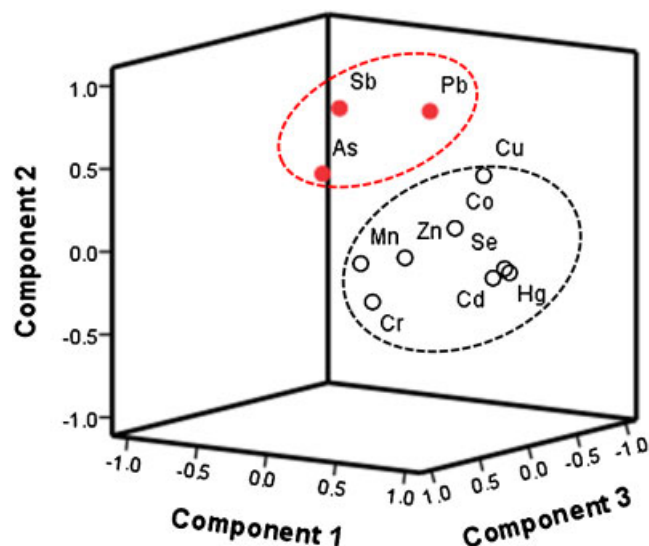


Fig. 3. Principal component analysis of hepatic elements. Two groupings are apparent: (1) As, Pb, and Sb and (2) all other elements including Hg. [Color figure can be seen in the online version of this article, available at [wileyonlinelibrary.com](http://www.wileyonlinelibrary.com).]

Table 4. Correlations between elemental concentrations and neurochemicals (enzymes and receptors) in the brains of wild bald eagles (*Haliaeetus leucocephalus*)^a

	GS activity (n = 44)		GAD activity (n = 41)		NMDAR binding (n = 46)		GABA(A)R binding (n = 46)	
	r	p	r	p	r	p	r	p
Cr	0.137	0.375	-0.161	0.314	-0.166	0.271	0.093	0.537
Mn	0.148	0.339	0.252	0.112	-0.192	0.201	0.116	0.444
Co	0.124	0.421	-0.361	0.020	0.252	0.091	-0.034	0.824
Cu	0.323	0.033	-0.105	0.515	0.106	0.484	-0.012	0.935
Zn	0.375	0.012	-0.162	0.311	0.118	0.436	0.114	0.449
As	0.085	0.585	-0.291	0.065	-0.205	0.171	0.094	0.533
Cd	0.086	0.580	-0.375	0.016	0.058	0.704	-0.187	0.213
Sb	0.04	0.798	-0.061	0.704	-0.192	0.202	0.236	0.114
Pb	-0.051	0.744	-0.342	0.029	0.202	0.179	0.293	0.053
IHg	0.188	0.030	-0.196	0.030	-0.282	0.001	0.026	0.771

^aInorganic mercury (Hg) data were obtained from the study of Rutkiewicz et al. [8].

GS = glutamine synthetase; GAD = glutamic acid decarboxylase; NMDA = *N*-methyl-D-aspartate; GABA(A) = γ -aminobutyric acid A.

bioaccumulation and its potential risks to wild birds. A recent study shows that hepatic Sb levels in tinamou species from a polluted site (for example, a mining area) ranged from 0.03 to 0.20 ppm (mean, 0.06 ppm), and that Sb exposure in these birds was 10 to 20 times higher than in control birds [33]. Despite tremendous variations of hepatic Sb in the bald eagles from the present study, 19.5% of birds had more than 0.20 ppm in the liver, which was the highest Sb level found in the mine-exposed tinamou.

There were significant correlations among toxic elements (Pb, Cd, Hg, Cr, or As) within the liver and within the brain (Tables 2 and 3). In addition, some toxic elements, such as As, Cd, and Pb, were positively correlated between the liver and

brain (Fig. 1). Although further studies are required to resolve exposure pathways of each element, at least in some parts, the data presented here show elevated exposure to toxic Pb and Sb (including relatively low As) that may be associated with Pb-shot ingestion. This is supported by examining the outcome of our principal component analysis (Fig. 3), in which two eigenvalues explained 45.4% of the total variance and resulted in two groups, the first consisting of Pb, Sb, and As, and the other comprising all other elements. By including Hg data from these same birds [8], we identify both Pb and Hg as being present at high levels in some cases (10.8% of birds had toxic levels of both heavy metals). Both are ubiquitous pollutants in the Great Lakes ecosystem. From the principal component analysis, the

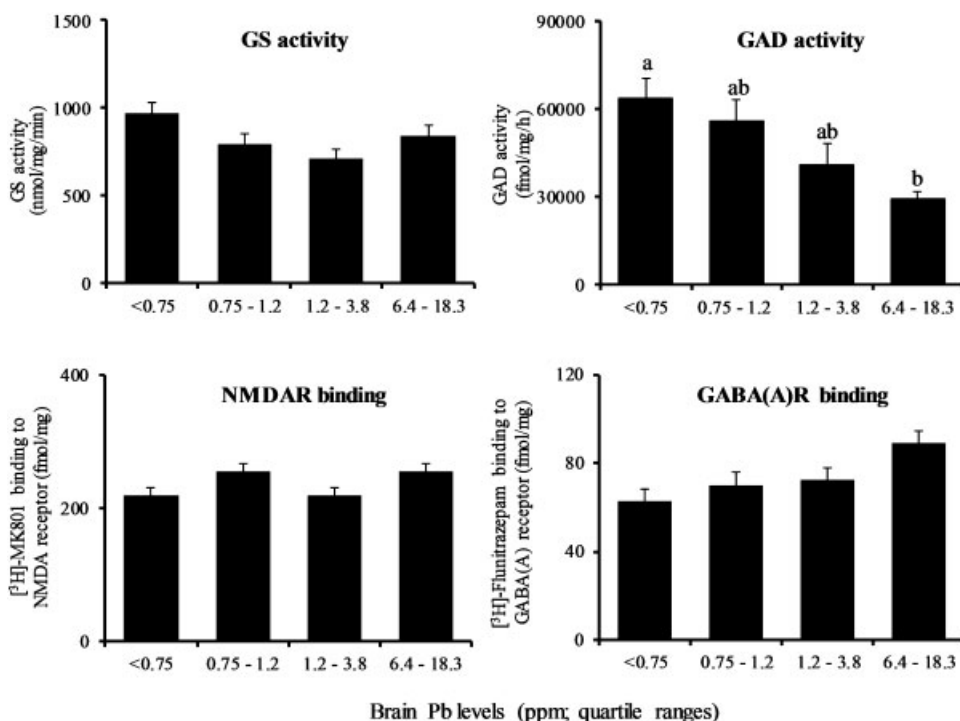


Fig. 4. Mean (\pm standard error) neurochemical enzyme activities and receptor levels in bald eagles (*Haliaeetus leucocephalus*) separated into brain quartiles of Pb exposure ($n = 10$ or 11 per group for glutamine synthetase [GS] and glutamic acid decarboxylase [GAD] activities; $n = 11$ or 12 per group for *N*-methyl *D*-aspartate [NMDA] and γ -aminobutyric acid A [GABA(A)] receptor levels). Letters denote significant differences ($p < 0.05$) among the bars following one-way analysis of variance.

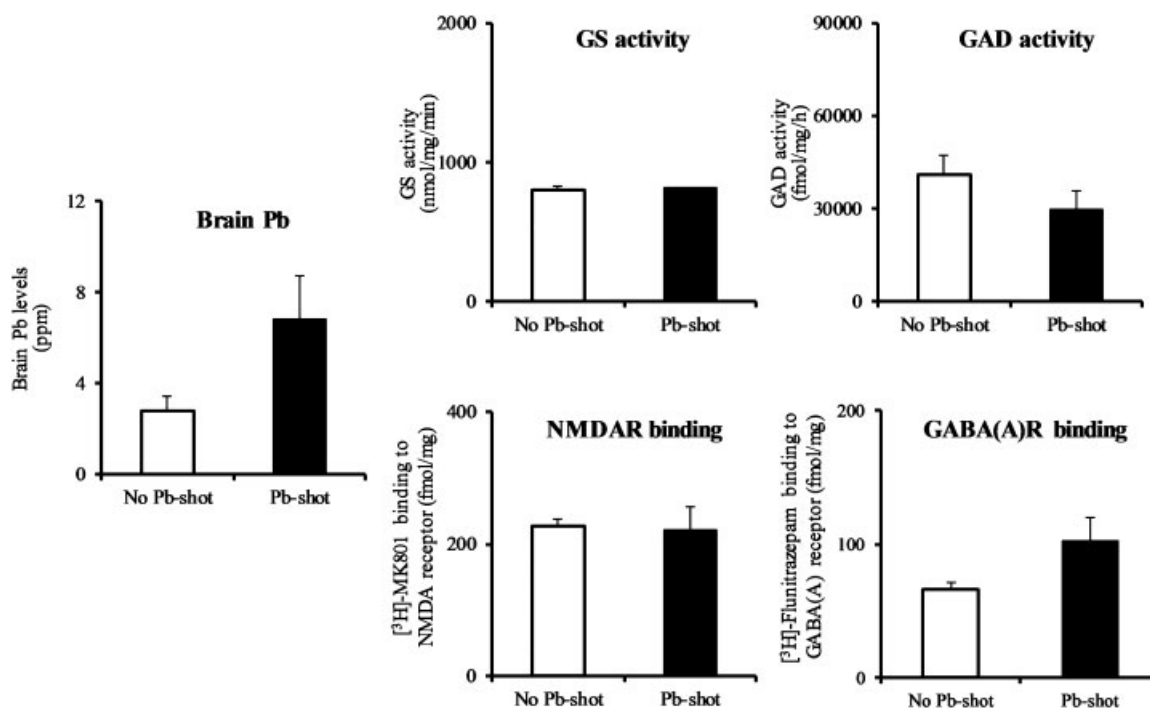


Fig. 5. Comparisons of brain Pb levels and four neurochemical biomarkers (mean \pm standard error) between Pb-shot–negative ($n = 18$) and Pb-shot–positive ($n = 8$) cases from Michigan bald eagles (*Haliaeetus leucocephalus*). GS = glutamine synthetase; GAD = glutamic acid decarboxylase; NMDA = N-methyl D-aspartate; GABA(A) = γ -aminobutyric acid A.

source-exposure routes for Pb may be differentiated from Hg in bald eagles (likely Pb shot ingestion vs fish consumption). Both Pb and Hg are proven neurotoxicants, but little is known about their cumulative impacts on avian health.

The neurotransmitters glutamate and GABA have critical roles in vertebrate reproduction and neurobehavior [34]. Here, Pb-, Cd-, and Co-associated decreases in GAD activity were found (Table 4). Because of high Pb exposure and its neurotoxic potential, the eagles were stratified into quartiles according to brain Pb levels. For GAD, the difference in mean enzyme activity between the lowest (<0.75 ppm) and highest (>6.4 ppm) Pb quartile was 54.2% (Fig. 4). Reduction in GAD activity has been observed in Pb-exposed organisms [34].

For the GABA(A) receptor, we found a 42% increase in receptor density in the highest (>6.4 ppm Pb) quartile when compared to the lowest (<0.75 ppm Pb) quartile (Fig. 4). There was also a positive correlation between GABA(A) receptor levels and brain Pb levels (Table 4; $r = 0.293$, $p = 0.053$); however, his correlation was not to a level of statistical significance. Although Co, Cd, and Hg were negatively associated with GAD activity (Table 4), no increasing trends of GABA(A) receptor levels were found by quartile analysis (data not shown). Increases in GABA(A) receptor density have been documented in response to a Pb-induced inhibition of GABA release in rodents [35]. In the present study, the GAD inhibition by aforementioned metals (Table 1), as well as a significant negative correlation between GAD activity and GABA(A) receptor levels ($r = -0.352$, $p < 0.05$), suggests that GABAergic components in wild bald eagles may be modulated following exposures to environmental elements, especially Pb.

Glutamine synthetase catalyzes the metabolic degradation of glutamate to glutamine. This enzyme, together with glutamate uptake transporters, tightly regulates extracellular glutamate to ensure homeostasis and prevent glutamate-mediated excitotoxic damage. We have recently completed a series of studies

(in vitro using isolated synaptosomes, field-based collection of brain tissues, and controlled laboratory bioassays) across several species of fish, birds, and mammals to document that environmentally relevant levels of Hg can inhibit glutamate uptake (D-H. Nam and N. Basu, University of Michigan, unpublished data), which would lead to a build-up of glutamate in the synapse. Our present (Table 4) and previous results [8] showed that GS activity significantly increased in association with brain Zn, Cu, and Hg. In mammals, high concentrations of extracellular glutamate, which is neurotoxic, increases GS expression, resulting in increased uptake of glutamate into astrocytes [36]. Though Zn and Cu are essential metals in the brain, Zn (>100 μ M) and Cu (25 μ M) can inhibit astrocyte glutamate uptake by poly(ADP-ribose) polymerase-1 activation [37] and hydroxyl radicals [38], respectively. Increases in GS activity are likely an adaptive cellular response to Zn, Cu, and Hg exposure to ensure that synaptic glutamate levels remain within a physiological range and do not lead to excitotoxic damage.

The NMDA receptor is the main excitatory neurotransmitter receptor in the vertebrate nervous system [39]. In the present study, we observed no relationship between concentrations of brain elements and NMDA receptor levels (Table 4). However, in these same bald eagles, we had previously found a significant decrease in NMDA receptor levels ($n = 128$, $r = -0.245$, $p < 0.01$) associated with brain total Hg (mean: 2.8 ppm; range: 0.2–34 ppm) (Table 4; [8]). A negative correlation has also been found between Hg and NMDA receptor levels in a range of wild (e.g., mink, polar bear, bald eagle) and laboratory (e.g., captive mink) animals [7,16,17]. These results suggest that the decrease in NMDA receptor levels may represent a compensatory scheme to cope with Hg-associated excitotoxic damage.

An important limitation of this work is the recognition that neurochemical biomarkers vary in their postmortem stability. We have addressed this previously [40], and in a forthcoming

laboratory-based study we have specifically addressed the four biomarkers studied here (J. Rutkiewicz and N. Basu, University of Michigan, unpublished data). In general, [³H]MK-801 binding to the NMDA receptor was quite stable under all post-mortem conditions tested (temperature, time, freeze-thaw cycles), GAD activity exhibited some stability, and binding to the GABA(A) receptor and GS activity exhibited poor stability. These postmortem stability data need to be considered when interpreting the results presented in the present study.

In conclusion, our results show that bald eagles are exposed to various elements, especially Pb and Hg, that may be present at toxic levels to a sizeable number of birds. The concentrations found in brain and associations with neurochemical biomarkers suggest that levels may be sufficient to alter GABAergic and glutamatergic signaling. Because of the fundamental roles of these neurotransmitter pathways in vertebrate neurobehavior and reproduction, prolonged neurochemical alterations may impact wildlife health. These findings raise further questions about the ecological and physiological impacts of metals in the environment and their impact on wild bald eagle populations.

Acknowledgement—We thank collaborators within the Michigan Department of Natural Resources and Environment (T. Cooley), the University of Minnesota Raptor Center (I. Bueno Padilla), and the Minnesota Department of Natural Resources (L. Shen, R. Bhagyam) for their assistance in the collection and dissection of eagle carcasses. J. Rutkiewicz was funded by a University of Michigan Regents Fellowship. Funding for the study was provided by grants to N. Basu from the University of Michigan School of Public Health and the Great Lakes Air Deposition program.

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