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Mercury, selenium and neurochemical biomarkers in different brain regions of migrating common loons from Lake Erie, Canada

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Abstract Common loons (*Gavia immer*) can be exposed to relatively high levels of dietary methylmercury (MeHg) through fish consumption, and several studies have documented MeHg-associated health effects in this species. To further study the neurological risks of MeHg accumulation, migrating loons dying of Type E botulism were collected opportunistically from the Lake Erie shore at Long Point (Ontario, Canada) and relationships between total mercury (THg), selenium (Se), and selected neurochemical receptors and brain enzymes were investigated. THg concentrations were 1–78 μg/g in liver; and 0.3–4 μg/g in the brain (all concentrations reported on a dry weight basis). A significant ($p < 0.05$) positive correlation was found between THg in liver and THg in 3 subregions of the brain (cerebral cortex: $r = 0.433$; cerebellum: $r = 0.293$; brain stem: $r = 0.405$). THg varied significantly among different brain regions, with the cortex having the highest concentrations. Se levels in the cortex and cerebellum were 1–29 and 1–10 μg/g, respectively, with no significant differences between regions. Se was not measured in brain stem due to insufficient tissue mass. There were molar excesses of Se over mercury (Hg) in both cortex and cerebellum at all Hg concentrations, and a significant positive relationship between THg and the Hg:Se molar ratio (cortex: $r = 0.63$; cerebellum: $r = 0.47$). No significant associations were observed between brain THg and the N-methyl-D-aspartic acid (NMDA) receptor concentration, nor between THg and muscarinic cholinergic (mACh) receptor concentration; however, brain THg levels were lower than in previous studies that reported significant Hg-associated changes in neuroreceptor densities. Together with previous studies, the current findings add to our understanding of Hg distribution in the brain of common loons, and the associations between Hg and sub-lethal neurochemical changes in fish-eating wildlife.

Keywords Mercury · Selenium · Common loon · Neurotransmitters · Great Lakes · Neurotoxicology · Wildlife

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Introduction

Wild fish-eating birds are at relatively high risk for elevated dietary methylmercury (MeHg) exposure, as a result of biomagnification of MeHg through aquatic food chains (Scheuhammer et al. 2007). Numerous studies have demonstrated negative health effects of MeHg in various avian species, with impaired reproduction and behavioral changes being frequently reported. MeHg altered parental behavior in captive American kestrels (*Falco sparverius*) (Albers et al. 2007), resulting in an increased number of infertile eggs due to impaired mating coordination (Bennett et al. 2009). Captive great egret (*Ardea alba*) nestlings fed a diet containing MeHg displayed nervous system lesions and altered neurological function including tremors, posture changes, and uneven gait (Spalding et al. 2000). One of the best studied wildlife species with respect to toxic effects of environmentally realistic exposures to MeHg is the common loon (*Gavia immer*). In wild common loons, dietary MeHg exposure has been associated

with behavioral alterations resulting in reduced productivity and altered parent–chick relationships (Barr 1986; Burgess and Meyer 2008; Evers et al. 2008), and in captive dosing studies, dietary MeHg exposure caused immunosupression (Kenow et al. 2007) and changes in loon behavior (Kenow et al. 2010) Many of these effects were observed following exposure of birds to levels of dietary MeHg that are relevant in the Great Lakes ecosystem.

Although MeHg is well known as a potent neurotoxicant, its specific effects on neurochemical pathways have only recently begun to be studied in wild avian species (Scheuhammer et al. 2008; Rutkiewicz et al. 2010, 2011). Neurochemical changes may provide early indication of future structural and functional damage to the central nervous system and thus monitoring neurochemical changes represents a potential risk assessment tool in ecotoxicology (Adams et al. 2010; Basu et al. 2005, 2007; Gagné et al. 2007; Nam et al. 2010). In addition, despite experimental evidence that selenium (Se) may afford protection against the neurotoxicity of MeHg, the possible modulating effects of Se on MeHg-induced neurochemical changes in free-ranging wildlife are poorly understood (Scheuhammer et al. 2008). Thus, we undertook the present study to assess Hg and Se concentrations, and various neurochemical parameters, in an opportunistically collected sample of common loons found dead of botulism on the north shore of Lake Erie, Ontario, Canada.

Methods

Carcasses of common loons ($n = 68$) dying of botulism were collected opportunistically during fall migration in November 2005, near Long Point, Ontario, Canada, shipped frozen, using storage and handling procedures as previously described (Stamler et al. 2005), to the National Wildlife Research Centre (NWRC), Environment Canada, Ottawa, Canada, and stored frozen until analysis. Livers ($n = 68$) were excised, and based on a range of THg concentrations analyzed in liver tissue, a subset of loons were chosen for brain excision ($n = 41$; 20 female and 21 male). Brains were dissected into 3 separate regions-brain stem, cerebellum, and cortex—and were stored at −80°C in separate acid washed polyethylene vials prior to preparation for Hg, Se, and neurochemical analyses. These three brain regions were chosen based on previous wildlife studies showing them to be sensitive to MeHg (Basu et al. 2007, 2010).

Hg and Se analysis

Liver and brain samples were prepared and analyzed for THg and Se in an accredited NWRC laboratory for metals analyses using established protocols as described previously (Scheuhammer and Bond 1991; Scheuhammer et al. 1998; Neugebauer et al. 2000; Weech et al. 2004). THg concentrations were measured in dried tissue homogenates using a Direct Mercury Analyzer (DMA-80, Milestone Inc., CT). All elemental residue concentrations are reported on a dry weight basis unless otherwise indicated. Analytical accuracy and precision were monitored through the use of Standard Reference Materials (SRMs), and intermittent analysis of duplicate samples. SRMs included National Research Council of Canada (NRCC) DOLT-3 (dogfish liver), DORM-2 (dogfish muscle), and TORT-2 (lobster hepatopancreas). Average recovery of Hg and Se was within 10% of certified values for all analyses; similarly, analytical precision (% Relative Standard Deviation of replicate samples) averaged 10% for all analyses.

Neurotransmitter receptor assays

Concentrations of glutamate (*N*-methyl-D-aspartic acid-NMDA) and muscarinic acetylcholine (mACh) neuroreceptors were determined in each brain region. Cellular membranes were prepared from brain tissue using protocols described in detail elsewhere (Scheuhammer et al. 2008; Nam et al. 2010). For mACh receptor assays, a Na/K buffer was used (50 mm NaH_2PO_4, 5 mm KCl, 120 mm NaCl, pH 7.4) and for NMDA receptors, a Tris buffer was employed (50 mm Tris, 100 μm glycine, 100 μm L-glutamic acid, pH 7.4). 30 μg of prepared membrane was re-suspended in the appropriate buffer and added to microplate wells containing a 1.0 μm GF/B glass filter (Millipore, Boston, MA, USA). For mACh receptor binding, samples were incubated with 1 nm [^{3}H]-QNB (42 Ci mmol^{-1}; NEN/Perkin Elmer, Boston, MA, USA) for 60 min. For NMDA receptor binding, samples were incubated with 5 nm [^{3}H]-MK-801 (22 Ci mmol^{-1}; NEN/Perkin Elmer) for 120 min. All assays were carried out on a shaking platform at room temperature. Binding reactions were terminated by vacuum filtration. The filters were rinsed three times with buffer and then allowed to soak for 96 h in 25 μl of OptiPhase Supermix Cocktail (Perkin Elmer). Radioactivity retained by the filter was quantified by liquid scintillation counting in a microplate detector (Wallac Microbeta, Perkin Elmer) having a counting efficiency of approximately 35%. Specific binding to both receptors was defined as the difference in radioligand bound in the presence and absence of 100 μM unlabelled atropine (for mACh receptors) and MK-801 (for NMDA receptors). Binding was reported as fmol of radioisotope bound per mg of membrane protein (fmol/mg). All samples were assayed in quadruplicate for total and non-specific binding. Intra- and inter-plate variation in binding was less than 10% as determined by use of internal, pooled controls.

Brain enzyme analysis

The activities of cholinesterase (ChE) and monamine oxidase (MAO) were determined as previously described (Scheuhammer et al. 2008; Nam et al. 2010). Briefly, tissues were sonicated for 30 s in cold Na/K buffer including 0.5% (v/v) Triton X-100. Following a 10 min centrifugation at 15,000*g* (4°C), the supernatant was removed. For ChE activity, 0.5 μg of supernatant protein was mixed with 100 μM 10-acetyl-3,7-dihydroxyphenoxazine, 200 mU horseradish peroxidase, 20 mU choline oxidase, and 100 μM acetylcholine. For MAO activity, 5 μg of protein was mixed with 100 μM 10-acetyl-3,7-dihydroxyphenoxazine, 200 mU horseradish peroxidase, and 100 mM tyramine. Following a 30 min incubation period for assays, the reaction end-product, resorufin (ex = 540, em = 590), was monitored between 30 and 60 min (CytoFluor 2350, Millipore, Bedford, MA, USA). Specific activities of ChE and MAO were expressed as nmol resorufin formed per min per unit (μg or mg) protein. Each sample was assayed in triplicate. Intra- and inter-plate variation in binding was less than 7% as determined by use of internal standards.

Statistical analyses

For all statistical comparisons, the critical level of significance was set at $\alpha = 0.05$. THg results are reported as mean ± standard deviation. Prior to statistical analysis, brain and liver THg levels were log-transformed to achieve a normal distribution (determined using the method of D'Agostino et al. (1990). One-way ANOVAs were used to test whether concentrations of THg, Se, or neurochemical biomarkers varied significantly across the three brain regions, except for NMDA and MAO for which the non parametric Kruskal–Wallis test was used as log transformation was unable to normalize these neurochemical data. Standard linear correlation analyses were used to test whether there were significant associations between THg and any of the neurochemical variables, Standard linear correlation analyses were also used to test whether THg in liver was significantly correlated with THg in any of the three brain regions; and also to test if THg and Se were significantly correlated in any of the three brain regions. All statistical analyses were performed using SAS (Version 9.1, Institute, Cary, NC, USA).

Results

THg in common loon liver tissue ranged from 1.17 to 77.2 μg/g, with a mean of 22.8 μg/g (Table 1). The brain regions revealed substantially lower THg concentrations than the liver, ranging from 0.27 to 3.43 μg/g in the brain stem, 0.32–2.83 μg/g in the cerebellum, and 0.37–3.65 μg/g in the cortex, with overall means of 1.16, 1.17, and 1.38 μg/g, respectively (Table 1). There were significant differences in THg between the three regions (Table 1), determined by ANOVAs performed on log-transformed data, with THg levels in the cortex significantly higher than

Table 1 Total Hg and Se concentrations (means ± SD; ug/g dry wt.), Hg:Se molar ratios, muscarinic (mACh) and glutamate (NMDA) receptor levels (means ± SD; fmol/mg protein), and cholinesterase (ChE) and monoamine oxidase (MAO) enzyme activity (means ± SD; nM/min/μg protein) for three brain regions from common loons found dead of botulism

	Cortex	Cerebellum	Brainstem
THg	1.38 ± 0.72 (41)[a]	1.17 ± 0.57 (41)[b]	1.16 ± 0.71 (41)[b]
	Range 0.37–3. 65	Range 0.32–2.83	Range 0.27–3.43
Se	5.22 ± 4.22 (41)	4.27 ± 2.21 (41)	Se not analyzed
	Range 1.47–28.49	Range 1.07–10.09	
Hg:Se molar ratio	0.15 ± 0.12 (40)	0.14 ± 0.11 (32)	Se not analyzed
	Range 0.02–0.58	Range 0.02–0.54	
mACh	109 ± 17 (41)[a]	107 ± 36 (41)[a]	82 ± 31 (41)[b]
	Range 38.5–187	Range 66.22–210.07	Range 75.33–147.17
NMDA	238 ± 66 (41)[a]	68 ± 42 (41)[c]	101 ± 51 (41)[b]
	Range 73.8–386	Range 13.8–189	Range 14.0–201
MAO	1.77 ± 1.22 (41)[a]	1.02 ± 0.67 (41)[b]	0.76 ± 0.54 (41)[b]
	Range 0.01–3.94	Range 0.01–2.32	Range 0.01–1.86
ChE	33.4 ± 17.1 (41)[a]	21.5 ± 15.0 (41)[b]	15.4 ± 11.8 (41)[b]
	Range 0.06–62.5	Range 0.09–56.2	Range 0.05–39.9

Bracketed values are sample sizes. Significant ($p < 0.05$) differences across brain regions for a given measurement (i.e., from results of ANOVAs comparing within rows) are indicated by superscript letters. Se was not determined for brainstem due to lack of adequate tissue mass

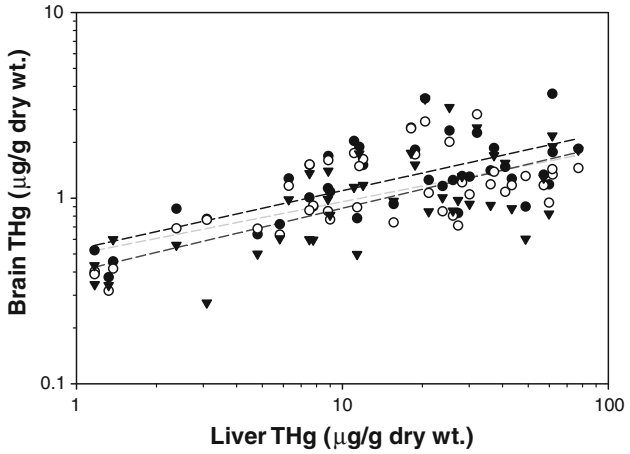

Fig. 1 Relationships between THg in liver and THg in three brain regions [Cortex (*black circle*); Cerebellum (*white circle*); Brain stem (*black triangle*)] from common loons found dead of botulism. *Dashed lines* represent best-fit linear regressions. Linear correlation coefficients (r), slopes, and y-intercepts for log-transformed data are similar for the 3 brain regions. Cortex: $r = 0.704$ ($p < 0.05$), slope = 0.32, intercept = −0.28; Cerebellum: $r = 0.657$ ($p < 0.05$), slope = 0.28, intercept = −0.30; brain stem: $r = 0.668$ ($p < 0.05$); slope = 0.34; intercept = −0.40

the two other regions. Concentrations of liver THg were positively correlated with THg in each of the brain regions, with the strongest association found between liver and cortex (Fig. 1).

Se levels ranged from 1.47 to 28.5 µg/g in the cortex; values were lower in the cerebellum but not to a level of statistical significance (Table 1). When compared to THg values from the same brain region, there were no significant correlations between Hg and Se. The molar ratio of Hg:Se ranged from 0.02 to 0.54 in the cerebellum, and 0.02–0.58 in the cortex, with overall mean Hg:Se molar ratio of 0.14 and 0.15, respectively (Table 1), indicating a molar excess of Se over Hg in all samples. Significant positive correlations were found in both regions between the Hg:Se molar ratios and THg ($p > 0.05$; Table 2 and Fig. 2), with a stronger association found in the cortex.

Four neurochemical biomarkers were measured in each of the three brain regions. For each of the biomarkers, significant differences in mean values were found across brain regions with levels generally greatest in the cerebral cortex (Table 1). However, no significant correlations were found between brain THg and neurochemical biomarkers in any of the three brain regions (Table 2). For the NMDA receptor, negative trends were found with THg in each of the three brain regions, but not to a level of statistical significance.

Discussion

As a piscivorous species with a wide geographic range, the common loon has been proposed as a useful bioindicator of dietary MeHg availability in aquatic environments across North America (Evers 2006). Studies on wild loons have documented associations between MeHg exposure and

Table 2 Linear correlations among THg in 3 brain regions and THg in liver, neuroreceptor density (mACh and NMDA), cerebral enzyme activity (ChE and MAO), Se concentrations in cortex and cerebellum, and Hg:Se molar ratios in cortex and cerebellum (selenium was not measured in brain stem due to insufficient tissue mass)

	Liver THg	mACh receptor	NMDA receptor	ChE activity	MAO activity	Cortex Se	Cortex Hg:Se molar ratio	Cerebellum Se	Cerebellum Hg:Se molar ratio
Cortex THg	$r = 0.70$ $p < 0.05$ Slope = 0.32 Intercept = −0.28	$r = -0.17$ $p > 0.05$	$r = -0.05$ $p > 0.05$	$r = 0.05$ $p > 0.05$	$r = 0.04$ $p > 0.05$	$r = 0.02$ $p > 0.05$	$r = 0.78$ $p < 0.05$ Slope = 1.14 Intercept = −1.05		
Cerebellum THg	$r = 0.66$ $p < 0.05$ Slope = 0.28 Intercept = −0.30	$r = -0.02$ $p > 0.05$	$r = -0.06$ $p > 0.05$	$r = 0.09$ $p > 0.05$	$r = -0.06$ $p > 0.05$			$r = 0.02$ $p > 0.05$	$r = 0.63$ $p < 0.05$ Slope = 1.09 Intercept = −0.98
Brainstem THg	$r = 0.67$ $p < 0.05$ Slope = 0.34 Intercept = −0.40	$r = 0.12$ $p > 0.05$	$r = -0.08$ $p > 0.05$	$r = 0.16$ $p > 0.05$	$r = 0.22$ $p > 0.05$				

Statistically significant ($p < 0.05$) relationships are also plotted graphically (Figs. 1, 2)

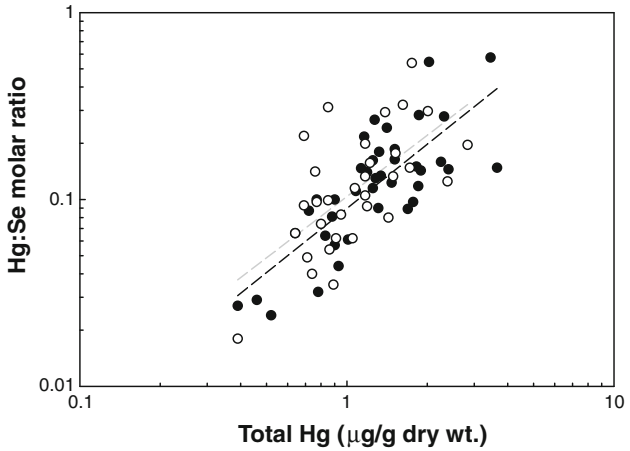

Fig. 2 Relationships between THg and Hg:Se molar ratio in Cortex (*black circle*) and Cerebellum (*white circle*) from common loons found dead of botulism. *Dashed lines* represent best-fit linear regressions. Linear correlation coefficients (r), slopes, and y-intercepts for log-transformed data are similar for both brain regions. Cortex: $r = 0.781$ ($p < 0.05$), slope = 1.14, intercept = −1.05; Cerebellum: $r = 0.632$ ($p < 0.05$), slope = 1.09, intercept = −0.98

behavioral alterations resulting in reduced reproductive capacity and altered parent–chick relationships (Nocera and Taylor 1998; Evers et al. 2008). A study by Evers et al. (2008), based on an extensive 18-years dataset of over 5,000 measurements, documented lethargy, altered reproductive behavior, and wing area asymmetry in loons related to MeHg exposure. Laboratory studies have confirmed some of these field observations, and have documented MeHg-associated oxidative stress (Kenow et al. 2008), immunosupression (Kenow et al. 2007), and changes in loon behavior (Kenow et al. 2010). Collectively, studies on wild and captive loons suggest that loons may be at particularly high risk for MeHg toxicity. Barr (1986) and Burgess and Meyer (2008) concluded that reproductive success in free-living loons was reduced by 50% when birds feed on fish with 0.2–0.3 μg/g Hg (wet wt.), and virtually no reproductive success was observed when birds feed on fish with 0.3–0.4 μg/g Hg (wet wt.), concentrations that are frequently observed in several fish species across eastern North America (Kamman et al. 2005).

Because common loons can be exposed to relatively elevated dietary concentrations of MeHg, methods are needed to determine levels of exposure that are associated with early and sub-clinical health effects (i.e., effects that precede overt gross toxicity). Neurochemical biomarkers may serve this purpose. Studies on fish and aquatic invertebrates (Gagné et al. 2007; Adams et al. 2010; Nam et al. 2010) and some fish-eating mammals (Basu et al. 2005; Basu et al. 2007) have shown that contaminant-associated neurochemical changes precede functional and structural damage to the brain. Similarly, Scheuhammer et al. (2008) documented Hg-associated neurochemical changes in wild common loons and bald eagles found dead in various locations across Canada; specifically, brain Hg levels were positively correlated with mACh receptors and negatively correlated with NMDA receptors. One limitation of the Scheuhammer et al. (2008) study was that Hg accumulation and neurochemical changes were not characterized in distinct brain regions, rather they were measured in the whole brains. The brain is perhaps the most heterogeneous organ in higher vertebrates and resolving Hg accumulation and associated changes in distinct anatomical regions may increase our understanding of the causative linkages between Hg concentrations, neurochemical changes, and observed behavioral changes. Accordingly, a major goal of the present study was to characterize THg levels and selected neurochemical parameters in three discrete brain regions previously shown to be affected by Hg in wildlife (Basu et al. 2007, 2010). In the current study, Hg concentrations varied across three common loon brain regions and were highest in the cerebral cortex. The observed range of brain Hg concentrations (0.3–3.7 μg/g across three brain regions) was much narrower than noted in a previous study on common loons found dead in Canada, in which whole brain Hg values ranged from 0.2 to 68 μg/g (Scheuhammer et al. 2008). The values reported in the present study, however, were similar to values reported for Hg in brains of Great Lakes herring gulls, and for some other aquatic bird species from non-contaminated sites in North America (Rutkiewicz et al. 2010).

In the current study, no significant Hg-associated changes in neurochemical biomarkers were observed. Similarly, Rutkiewicz et al. (2010) noted no significant associations between brain Hg and neurochemical markers in herring gulls; nor in bald eagles with relatively low concentrations of brain Hg (Rutkiewicz et al. 2011). We hypothesize that brain THg levels in these studies were probably too low to be associated with significant neurochemical change. The maximum loon brain Hg value measured in the current study is nearly 20-times lower than the maximum value reported by Scheuhammer et al. (2008) in which significant Hg-related neurochemical changes were found in loons. Taken together, these studies suggest that brain Hg concentrations <3 μg/g (dry wt.) may not be sufficiently high to produce consistent changes in brain neurochemistry in free-living avian species. In addition, loon brain samples analyzed in the current study for both THg and Se showed molar excesses of Se over Hg, and also exhibited significant positive correlations between Hg:Se and THg (Fig. 2). Excess molar concentrations of Se are generally thought to be protective against Hg toxicity (Ralston et al. 2008). The relatively low brain Hg concentrations that we observed coupled with molar excesses of Se in the same brain samples are consistent with a lack of Hg-related neurochemical effects.

Though a Hg-associated neurochemical effect threshold has not been established for wildlife, previous studies fish-eating mammals (Basu et al. 2006, 2007) and birds (Scheuhammer et al. 2008) have suggested that neurochemical changes occur in the 3–5 ppm brain Hg range. Also, Hoffman et al. (2011) reported that brain THg concentrations averaging about 3 μg/g (dry wt.) in breeding adult Forster's terns (*Sterna forsteri*) were associated with evidence of increased lipid peroxidation, loss of protein bound thiols, and decreased activity of antioxidant enzymes. Effects thresholds may vary between bird species, and also between mammals and birds. In a study on polar bear brain stem tissues, Hg-associated decreases in NMDA receptors were reported despite brain Hg levels being less than 1 μg/g (Basu et al. 2009). In a study of laboratory mink fed MeHg, a Hg-associated decrease in NMDA receptor levels was found in the lowest exposure group (0.1 μg/g in diet resulting in brain Hg levels of 1–2.2 μg/g) (Basu et al. 2007). Although significant associations were not observed in the current study, a weak negative trend was observed between brain Hg levels and NMDA receptor levels. Results of the present study are valuable to help determine threshold tissue-Hg concentrations that are associated with significant changes in important neurochemical signaling pathways in MeHg-exposed wildlife.

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