Neurochemical Biomarkers to Assess Mercury's Health Impacts in Birds

by

Jennifer M. Rutkiewicz

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Doctoral Committee:

Assistant Professor Niladri Basu, Chair
Professor G. Allen Burton
Professor Robert Denver
Professor Martin Philbert

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Table of Contents

Acknowledgements ii

List of Figures viii

List of Tables ix

List of Abbreviations x

Abstract xi

Chapter 1 Introduction 1
- 1.1 Background 1
  - 1.1.1 Mercury Overview 1
  - 1.1.2 Mercury in Wild Birds 4
  - 1.1.3 Wild Avian Sentinels 6
  - 1.1.4 Neurochemical Biomarkers 7
- 1.2 Knowledge Gaps 12
- 1.3 Objective 13
- 1.4 Hypothesis 14
- 1.5 Specific Aims 15
- References 18

Chapter 2 Investigation of spatial trends and neurochemical impacts of mercury in herring gulls across the Laurentian Great Lakes 25
- 2.1. Introduction 25
- 2.2. Methods 27
  - 2.2.1 Animals and Study Area 27
  - 2.2.2 Hg Analysis 28
  - 2.2.3 [3H]-Ligand Binding to Neurochemical Receptors 28
  - 2.2.4 Expression of nAChR α-7 mRNA 29
  - 2.2.5 Statistical Analyses 30
- 2.3 Results 31
- 2.4 Discussion 32
- 2.5 Conclusions 35

Chapter 3 Mercury exposure and neurochemical impacts in bald eagles across several Great Lakes states 45

3.1 Introduction 45
- 3.2 Methods 47

3.2.1 Animals 47
3.2.2 Hg Analysis 48
3.2.3 Se Analysis 49
3.2.4 Neurochemical Assays 50
3.2.5 Statistical Analyses 52
3.3 Results 52
3.3.1 Brain Hg and Se 52
3.3.2 Liver Hg and Se 53
3.3.3 Feather Hg 54
3.3.4 Neurochemical Assays 54
3.4 Discussion 55
Figures and Tables 61
References 64

Chapter 4 Distribution of mercury in the white leghorn chicken embryo and hatchling following air cell egg injection 68
4.1 Introduction 68
4.2 Methods 70
4.2.1 Animals 70
4.2.2 Hg Analysis 71
4.2.3 Statistics 72
4.3 Results 72
4.3.1 Tissue Distribution in Embryos 72
4.3.2 Tissue Distribution in Hatchlings 73
4.3.3 Tissue Distribution in Day 15 Quail Embryos 74
4.3.4 Methylmercury Cysteine and Mercury (2) Chloride Injection 75
4.4 Discussion 75
Figures and Tables 81
References 87

Chapter 5 Pathology, neurochemistry and behavior in the white leghorn chicken embryo and hatchling following air cell injection of methylmercury chloride 90
5.1 Introduction 90
5.2 Methods 94
5.2.1 Animals 94
5.2.2 Mercury Analysis 95
5.2.3 Histology 95
5.2.4 Neurochemistry 96
5.2.5 Behavior 97
5.2.6 Statistics 99
5.3 Results 99
5.3.1 Mortality Rates 99
5.3.2 Brain THg Concentrations 100
5.3.3 Histology 100
5.3.4 Neurochemistry 101
5.3.5 Behavior 102
5.4 Discussion 104

Figures and Tables 111
References 118

Chapter 6 Associations between brain mercury levels and neurochemistry in the thick-billed murre (*Uria lomvia*) embryo following methylmercury chloride egg injection 123
6.1 Introduction 123
6.2 Methods 125
6.2.1 Animals 125
6.2.2 Hg Analysis 126
6.2.3 Neurochemical Assays 126
6.2.4 Statistics 126
6.3 Results 127
6.3.1 Hg Analysis 127
6.3.2 Neurochemical Assays 127
6.4 Discussion 128
Figures and Tables 133
References 136

Chapter 7 Brain mercury and neurochemistry in young zebra finches (*Taeniopygia guttata*) exposed developmentally to methylmercury 139
7.1 Introduction 139
7.2 Methods 141
7.2.1 Animal Care 141
7.2.2 Food Preparation 142
7.2.3 Dosing 142
7.2.4 Tissue Collection 143
7.2.5 Mercury Analysis 143
7.2.6 Neurochemistry 144
7.2.7 Statistics 144
7.3 Results 145
7.3.1 Brain THg Concentrations 145
7.3.2 Neurochemistry 145
7.4 Discussion 146
Figures and Tables 150
References 153

Chapter 8 Postmortem stability of brain GABAergic and glutamatergic receptors and enzymes under ecological conditions 155
8.1 Introduction 155
8.2 Methods 158
8.2.1 Tissues 158
8.2.2 Postmortem Conditions 158
8.2.3 Neurochemical Assays 159
8.2.4 Statistical Analysis 160
8.3 Results 160
8.3.1 Effects of Postmortem Temperature and Time 160

8.3.2 Effects of Storage Temperature and Time 161
8.3.3 Effects of Freeze Thaw Cycles 162
8.4 Discussion 162
Figures 170
References 173

Chapter 9 Conclusion 176
9.1 Objective and Significance 176
9.2 Major Results and Discussion 178
9.2.1 Mercury Exposure and Neurochemistry in Juvenile and Adult Wild Birds 178
9.2.2 Mercury Exposure and Neurological Impacts in Laboratory Embryos and Hatchling Birds 182
9.2.3 Limitations of Neurochemical Biomarkers 187
9.3 Future Research 189
Figures and Tables 192
References 197

List of Figures

Figure 2.1 Total mercury (THg) levels in the brains of adult herring gulls collected from five Great Lakes colonies and one non-Great Lakes reference colony 37
Figure 2.2 Mercury and nerochemical receptor levels in herring gulls 38
Figure 3.1 Total mercury (THg) in brain and liver of wild bald eagles from Minnesota (MN), Iowa (IA), Wisconsin (WI), Michigan (MI), and Ohio (OH) (2002-2010) 61
Figure 3.2 Mercury and neurochemistry in bald eagles 62
Figure 4.1 Total mercury (THg) concentrations (μg/g wet weight) in tissues of chicken embryos and hatchlings from eggs injected with methyl-mercury chloride (6.4 μg/g egg) in corn oil on incubation day 0 81
Figure 4.2 Mercury distribution in chicken and quail embryos 82
Figure 4.3 Comparison of tissue mercury in chicken embryos after injection of MeHgCl, MeHgCys, or $HgCl_2$ 83
Figure 5.1 Neuropathology in cerebellum of day 19 chicken embryos 111
Figure 5.2 Brain mercury and neurochemistry in day 19 chicken embryos exposed to methylmercury chloride by egg injection 112
Figure 5.3 Brain mercury and neurochemistry in day 19 chicken embryos exposed to methylmercury cysteine by egg injection 113
Figure 6.1 Brain mercury and neurochemistry in pipping thick-billed murre embryos exposed to methylmercuy chloride by egg injection 133
Figure 7.1 Brain mercury and neurochemistry in 50 day old zebra finches exposed to methylmercury cysteine by maternal deposition 150
Figure 8.1 Effects of postmortem environmental conditions on neurochemical biomarkers in chicken brain 170
Figure 8.2 Effects of carcass storage conditions on neurochemical biomarkers in chicken brain 171
Figure 8.3 Effects of freeze thaw cycles on neurochemical biomarkers in chicken brain. 172
Figure 9.1 Schematic of the proposed progression and tiers of neurological change following exposure to mercury (Hg) 192
Figure 9.2 Summary of methylmercury (MeHg) exposure routes, avian species, and neurochemical findings from field and laboratory studies that are described in this dissertation 193

List of Tables

Table 2.1 Brain mercury and neurochemical receptor levels and expression in herring gulls 39
Table 2.2 Mean values (and range) of total mercury (THg) in the brains of fish-eating birds 40
Table 3.1 Mercury in bald eagle brain and liver 63
Table 4.1 Experimental conditions and sampling details for mercury egg (air cell) injection studies 84
Table 4.2 Mercury concentrations in chicken embryos after methylmercury chloride egg injection 85
Table 4.3 Mercury distribution in chicken brain 86
Table 5.1 Experimental conditions and sampling details for mercury egg (air cell) injection studies 114
Table 5.2 Mortality rates in white leghorn chicken and Japanese quail embryos in mercury egg (air cell) injection studies 115
Table 5.3 Neurochemical receptor and enzyme values in Japanese quail embryos 116
Table 5.4 Behavioral tests in chicken hatchlings 117
Table 6.1 Mercury in thick-billed murre pipping embryo brain. 134
Table 6.2 Neurochemical receptor and enzyme values in thick-billed murre pipping embryos 135
Table 7.1 Mercury in zebra finch brain 151
Table 7.2 Neurochemical receptor and enzyme values in zebra finches 152
Table 9.1 Summary of published field studies investigating relationships between brain mercury (Hg) and neurochemistry in fish eating birds 194
Table 9.2 Summary of findings from studies in this dissertation investigating relationships between brain mercury (Hg) and neurochemistry in embryos and hatchlings following in ovo exposure to MeHg 195
Table 9.3. Summary of laboratory studies investigating relationships between mercury exposure and behavior in birds 196

List of Abbreviations

d.w.	Dry Weight
CBL	Cerebellum
CER	Cerebrum
DMA	Direct Mercury Analyzer
NMDA	*N*-Methyl-D-Aspartic Acid
GABA	γ-Aminobutyric Acid
GAD	Glutamic Acid Decarboxylase
GS	Glutamine Synthetase
Hg	Mercury
$HgCl_2$	Mercury (II) Chloride
IHg	Inorganic Mercury
mACh	Muscarinic Acetylcholine
MeHg	Methylmercury
MeHgCl	Methylmercury Chloride
MeHgCys	Methylmercury Cysteine
MT	Metallothionein
nACh	Nicotinic Acetylcholine
OHg	Organic Mercury
OL	Optic Lobe
RSD	Relative Standard Deviation
SRM	Standard Reference Material
w.w.	Wet Weight

Abstract

Neurochemical Biomarkers to Assess Mercury's Health Impacts in Birds

Mercury (Hg) is a neurotoxicant that can be associated with changes in neurochemical receptors and enzymes. The hypothesis tested here is that Hg induces changes in neurochemical biomarkers involved in glutamate and γ-aminobutyric acid (GABA) neurotransmission that may warn of clinical and behavioral impacts in birds. This project examined associations between Hg and neurochemistry in birds exposed in the field, and between Hg and neurochemistry, neuropathology, and neurobehavior in birds exposed developmentally in the laboratory. It also evaluates postmortem conditions that impact biomarker stability.

In field studies, herring gulls and bald eagles displayed dissimilar Hg exposures, with brain total Hg (THg) levels reaching 2.0 μg/g dry weight in gulls and 34 μg/g in eagles. Gulls showed no associations between the NMDA or cholinergic receptors and THg, while eagles showed a positive association between glutamine synthetase (GS) and THg/inorganic Hg (IHg), negative association between the NMDA receptor and THg/IHg, and negative relationship between glutamic acid decarboxylase (GAD) and IHg.

In the laboratory, white leghorn chickens exposed to Hg via egg injection showed no consistent associations between biomarkers (NMDA, GABA receptors,

GS, GAD) and Hg in whole brain or discrete brain regions at embryonic days 10, 14, and 19 or posthatch days 1 and 7, despite brain THg levels reaching 21.6 µg/g dry weight. Neurobehavior (righting, balance, startle) and neuropathology did not relate to Hg. Japanese quail embryos showed no neurochemical changes, but thick-billed murre embryos displayed a negative association between THg and GAD and a positive association between THg and the NMDA receptor. Zebra finches exposed via maternal deposition displayed no neurochemical changes.

Postmortem stability of biomarkers under environmentally relevant conditions was evaluated in chicken embryos in the laboratory. The NMDA receptor and GAD were stable under most conditions, but the GABA receptor and GS displayed reduced binding and activity.

This work demonstrates that dietary Hg relates to neurochemistry in some wild birds, but there may but. developmental exposure may not impact neurological outcomes. This work helps identify Hg-associated neurochemical changes, provides information about effects of developmental exposure, and increases knowledge of impacts across several avian species, exposures, and timepoints.

Chapter 1

Introduction

1.1 Background

1.1.1 Mercury Overview

1.1.1.1 Source and Fate of Mercury

Mercury (Hg) is a naturally occurring element that is ubiquitous in the environment worldwide. It exists in and can cycle between elemental, inorganic, and organic forms (Clarkson et al. 2006). Distribution of Hg in the environment, as well as absorption, distribution, and toxicity in an organism is highly dependent upon the chemical form of the element. Potential sources of Hg exposure include dental amalgam fillings, Hg thermometers, antibacterial agents, and paint, but the primary source of exposure in humans and wildlife is the consumption of fish (ATSDR 1999).

Though Hg is naturally occurring, its distribution in the environment is affected by human activity. Of the 5500 metric tons released to the atmosphere annually, approximately 70% are a result of human activity (UNEP 2002) and levels in the environment have increased five fold since the industrial revolution (Lindqvist 1991, Travis et al. 1992, Zillioux et al. 1993). Mercury is most commonly released as a byproduct of coal burning, which releases elemental Hg vapor into the air (ATSDR 1999). Owing to its half life of one year, it may be transported great

distances before being wet or dry deposited (Lindberg et al. 2007). Therefore, Hg may be found in environments, such as the Arctic, with few anthropogenic sources (Hylander et al. 2006). In the air, elemental Hg vapor is oxidized to inorganic Hg, which reaches the earth via wet and dry deposition (Lindberg et al. 2007). In the soil, water, and sediment, this inorganic Hg is converted to organic methylmercury (MeHg) by microorganisms (Jensen et al. 1969). Unlike the inorganic forms, organic Hg may penetrate cell membranes and accumulate in fish (Cappon et al. 1981), where it remains for months to years (USEPA 1997). Methylmercury biomagnifies through aquatic food chains (Jaeger et al. 2009), with levels in top predators reaching 10-100 times those in fish (USEPA 1997). Consequently, there is great concern over the possible effects of chronic exposure to MeHg via fish consumption for both wildlife and humans (Dorea 2008).

1.1.1.2 General Overview of Mercury Toxicity

Because Hg has a high affinity for thiol groups on proteins, it has the potential to disrupt enzymes, receptors, and other proteins. As a result, Hg has been associated with a variety of toxic effects including gastrointestinal problems, nephrotoxicity, and neurotoxicity, which is the primary concern following Hg exposure (ATSDR 2009, Clarkson et al. 2006). Inorganic Hg cannot readily enter the brain, but absorbed metallic Hg may cross the blood brain barrier and become oxidized to inorganic Hg, which can remain in the brain for years (ATSDR 2009). Methylmercury , which is of particular concern because it is the form found in fish, readily crosses-the blood brain barrier via methionine transporters (Kerper et al.

1992) and has been shown to exert neurotoxic effects including pathological and behavioral changes (ATSDR 2009, Clarkson et al. 2006).

Pathologically, MeHg causes numerous brain lesions, reduced brain size, and neuronal damage or loss (Castoldi et al. 2008, Eto et al. 1978, Johansson et al. 2007). Effects in adults are usually restricted to the visual cortex and granule layer of the cerebellum, but damage in developing brains is more widespread and can cause severe mental and sensory disabilities (Castoldi et al. 2001, Johansson et al. 2007). Several parameters of neurotransmission, including receptors, transporters, enzymes, and neurotransmitter levels, are also adversely affected by MeHg (Cagiano et al. 1991, Johansson et al. 2008). Neurotoxicity is often apparent as mental, sensory, and behavioral problems. Laboratory animals exposed to MeHg display deficits in motor coordination, cognition, learning, and memory (Bennett et al. 2009, Cagiano et al. 1990). Victims of the Minamata disaster in Japan suffer from sensory deficits, weakness, and mental retardation (Mergler et al. 2007). Also, numerous epidemiological studies have identified relationships between maternal MeHg and memory, attention, and motor function (Castoldi et al. 2008, Mergler et al. 2007). An ongoing cohort study of children of whale consuming mothers in the Faroe Islands showed neurobehavioral deficits throughout childhood, while another cohort study of children of fish consuming mothers in the Seychelles only began to detect motor skills deficits as the children aged (UNEP 2002, Mergler et al. 2007). Disparities may be attributed to diet or test design, but an increased understanding of subclinical changes involved mechanistically in neurotoxicity might also help to explain apparent differences in outcomes.

1.1.2 Mercury in Wild Birds

1.1.2.1 Exposure

Due to MeHg's tendency to biomagnify in aquatic ecosystems, fish eating birds often carry high burdens in their tissues. Total brain Hg levels in North American western grebes and osprey averaged approximately 1μg/g (dry weight) (Elbert et al. 1998, DesGranges et al. 1998), while mean levels in bald eagles, common loons, black crowned night herons, snowy egrets, and double crested cormorants were slightly higher, ranging from 2.5 to 7.5mg/g (Henny et al. 2002, Scheuhammer et al. 2008, Sepulveda et al. 1998). Mean levels in brains of several species from known contaminated sites reached 5-56.3 μg/g (DesGranges et al. 1998, Henny et al. 2002). Although a threshold for Hg's neurological effects has not been derived, changes in brain neurochemistry have been observed in common loons and bald eagles with mean total Hg levels of 4.0μg/g and 3.0μg/g respectively (Scheuhammer et al. 2008). This suggests that even ordinary environmental Hg exposure may have subtle neurological effects in many species. Current levels of Hg contamination are an immense ecological threat, as numerous bird populations across North America are exposed to Hg levels believed to be of toxicological concern (USEPA 1997, Scheuhammer et al. 2007).

1.1.2.2. Neurobehavioral Effects

Associations between motor skills and MeHg have been identified in birds. In captive egrets fed MeHg dosed fish, a high dose of 5 μg/g caused tremors and affected righting response, coordination, gait, and flying ability (Spalding et al.

2000). Similarly in common loons, dietary Hg exposure as low as 0.4 µg/g was associated with impaired righting response (Kenow et al. 2010). Methylmercury also affected balance in American kestrels fed 6 and 12 mg/g MeHg (Bennett et al. 2007). Laboratory chickens exposed *in ovo* to 1 µg MeHg injected in to the yolk sac displayed impaired locomotor activity (Carvahlo et al. 2008).

Cognitive behavior is also associated with MeHg in birds. Captive egrets fed environmentally relevant doses displayed altered preening (Spalding et al. 2005), hunting, and shade seeking behavior (Bouton et al. 1999). Captive common loons exposed to MeHg *in ovo* had reduced responses to a frightening stimulus compared to controls (Kenow et al. 2011). In captive American kestrels, MeHg exposure altered egg incubation behavior (Albers et al. 2007). In wild loons, Hg was associated with neurobehavioral changes that were believed to affect reproductive and chick rearing ability (Evers et al. 2008, Nocera et al. 1998).

Reproduction is among the most sensitive endpoints with respect to Hg toxicity in birds. The behavioral and motor deficits identified in several species may impair the ability of adult birds to breed and successfully raise chicks. In the laboratory, parental dietary MeHg exposure decreased egg hatching and fledging success in captive American kestrels (Albers et al. 2007). In the field, Hg exposure has been associated with reproductive impairment in common loons (Burgess et al. 2008, Evers et al. 2008), snowy egrets (Hill et al. 2008) and white ibises (Heath et al. 2005). Because impaired reproductive success can potentially impact birds on a population level, it has become increasingly important to identify Hg's earliest, subclinical effects.

Although avian studies are limited, identification of changes in neurochemical biomarkers may predict neurological damage before it has progressed to clinical toxicity such as brain lesions and behavioral alterations. In one of the first studies to assess neurochemical biomarkers in birds, associations between total Hg exposure and neurochemical alterations were identified in wild common loons and bald eagles, which displayed Hg associated decreases in binding to the NMDA receptor and increases in binding to the muscarinic cholinergic receptor (Scheuhammer et al. 2008). Identification of additional changes in neurochemistry is important, because these biomarkers of neurochemical function may provide important and early warning of behavioral changes that may ultimately influence survival and reproduction in birds.

1.1.3 Wild Avian Sentinels

Because many wild animals share common responses and exposure routes to toxicants, they are often used to monitor environmental contaminants and warn of risk to humans (NRC 1991). Wildlife sentinels have predicted toxic effects of endocrine disruptors in the Great Lakes (Colborne et al. 1996) and of MeHg at Minamata Bay, where neurological impairment was observed first in wild animals before humans (Harada 1995). Studies of wild species give an indication of effects of realistic levels and mixtures of chemicals, and often a dose-response curve for a toxic endpoint can be obtained from data collected in wild animals (Fox 2001). Laboratory studies of some species can then help to establish causal associations. Wild animals may provide an early warning, because they typically have short

lifespans, may be exposed to higher levels of toxicants, and display toxicity sooner than humans. Many wild species share common physiological processes with humans and react similarly to toxicants, while those that differ in responses and physiologies can aid in determination of toxic mechanisms (Basu et al. 2009a). Considering that fish eating wildlife accumulate high levels of MeHg, they may serve as valuable sentinels to warn of its exposure, effects, and risk.

1.1.4 Neurochemical Biomarkers

1.1.4.1 Overview of Neurochemical Biomarkers

Before progressing to structural and clinical damage, the brain undergoes more subtle changes in neurochemistry. These changes in neurochemical receptors, enzymes, and transporters can be used as biomarkers that provide an indication of subclinical neurological damage (Manzo et al. 1996). Neurochemical pathways are an ideal endpoint for neurotoxicity studies because they are directly related to brain function, and they may offer an early and sensitive indication of change before the brain has been irreversibly damaged. Neurotransmission can be affected by toxicants at the level of neurotransmitter production or metabolism, transport, storage, release, or receptor binding (Fitsanakis et a. 2005, Manzo et al. 1996).

Neurochemical biomarkers have been successfully associated with Hg exposure in several wild species. Studies in wildlife exposed to environmentally relevant levels have found a negative association between Hg and NMDA receptor levels in mink (Basu et al. 2007), polar bears (Basu et al. 2009b), loons, and eagles (Scheuhammer et al. 2008). *In vitro*, MeHg inhibited radioligand binding to the

NMDA receptor in mink brain (Basu et al 2007). These neurochemical changes upon environmental exposure to Hg warn of neurological damage in wildlife and may predict associated neurobehavioral changes.

1.1.4.2 Glutamate and GABA: Excitatory and Inhibitory Neurotransmission

Glutamate is the main excitatory neurotransmitter in the brain, and glutamatergic signaling plays a major role in learning, memory (Ozawa et al. 1998), and motor coordination (Kadotani et al. 1996, Ozawa et al. 1998). On the other hand, GABA is the primary inhibitory neurotransmitter and also plays a role in learning and memory development (Reis et al. 2009). As GABAergic signaling serves as an opposing signal to glutamatergic signaling, proper neurotransmission depends upon a balance between the two systems, and problems with either can potentially result in excitotoxicity (Reis et al. 2009). Disruption of glutamate signaling has the potential for severe consequences, as overstimulation, or excitotoxicity, can kill neurons (Fitsanakis et al. 2005). To prevent excitotoxicity, both systems rely on similar mechanisms for neurotransmitter production, metabolism, storage, and release.

Receptors with sites recognizing glutamate include G-protein coupled metabotropic receptors, and the ion channel based *N*-methyl-D-aspartate (NMDA), a-amino-3-hydroxy-5-methyl-4-isoxaloleproprionic acid (AMPA), and kainate receptors. Binding of ligand to these receptors alters transport of Na^+ and Ca^{2+} through the ion channel (Meldrum et al. 2000). Receptor levels are regulated by expression changes, post translational modifications, or degradation (Reis et al.

2009). The concentration of glutamate at the synapse is maintained at a low level by its uptake into astrocytes by excitatory amino acid transporters (EAAT) (Gras et al. 2006). Within the astrocyte, glutamate is enzymatically converted to nontoxic glutamine by glutamine synthetase (Gras et al. 2006) and after being released through sodium coupled neutral amino acid transporters (SNAT) (Yin et al. 2007), glutamine can be taken up through SNATs on glutamatergic neurons (Yin et al. 2007). There, glutamine is converted to glutamate by the enzyme glutaminase (Fitsanakis et al. 2005). Using vesicular glutamate transporters (vGluT), the newly produced glutamate is taken up by in presynaptic vesicles within the neuron and its release is mediated by Ca^{2+} (Gras et al. 2006, Reis et al. 2009).

Glutamate, produced either by glutaminase or by GABA transaminase (GABA-T) in the GABA shunt, is a precursor of GABA synthesis. GABA is produced from glutamate in a reaction catalyzed by glutamic acid decarboxylase (GAD). Like glutamate, GABA is recognized by metabotropic and ionotropic receptors. Binding to ionotropic $GABA_A$ receptors results in an inhibitory post-synaptic current, or, in a few cases, an excitatory response. The $GABA_A$ receptors respond to low extracellular levels of GABA and are modulated by drugs including benzodiazepines and barbituates, resulting in enhanced receptor affinity to GABA. $GABA_B$ receptors are G protein coupled metabotropic receptors that cause long lasting inhibitory post-synaptic currents and inhibit release of GABA and other neurotransmitters (Reis et al. 2009). For this reason, prevention of excitotoxicity depends on proper GABAergic signaling. Uptake by neurons and glia prevents overstimulation, and the GABA is either packaged for used as is within neurons or is converted to glutamine then

glutamate within glia. Excess GABA is degraded in a reaction catalyzed by GABA transaminase (GABA-T) (Reis et al. 2009).

1.1.4.3 Glutamate and GABA in Avian Physiology

Although GABA and glutamate have not been as well studied in birds as in rodents, it is clear that both neurotransmitter systems play a role in avian brain development and behavior. The NMDA receptor is expressed during chick development (Jacobsson et al. 1998), and NMDA receptor agonists are potent teratogens that alter neuronal migration (Andaloro 1998). Glutamatergic signaling is particularly important for learning in chicks. Chick performance in a bead discrimination test of memory formation is impeded by NMDA receptor agonists and antagonists, GS inhibitors, and treatment with high doses of glutamate (Gibbs et al. 2008). Less information regarding the role of GABA in the developing chick brain is available, but GABA and related enzymes are present in the developing chick embryo (Kuriyama et al. 1968) at an earlier stage than other neurotransmitters (Bondy et al. 1977), and are believed to support brain development (Sato et al. 2009). GABA is also important for chick learning; intracranial injection of low doses of GABA in chicks inhibits memory formation (Gibbs et al. 2005). Ataxia and loss of coordination upon treatment of chicks with excess GABA demonstrate the role of proper GABA regulation for motor skill development (Scholes 1965). Based on these studies, it is likely that disruption of glutamatergic and GABAergic neurotransmission will interfere with cognition and motor skills in chicks.

1.1.4.4 Effects of Mercury on Glutamate and GABA Systems

In laboratory studies, Hg has been associated with alterations in glutamatergic neurotransmission. Astrocytes are particularly susceptible to MeHg toxicity (Aschner 1996, 2000). Decreased uptake of glutamate by astrocytes is a hallmark of MeHg excitoxicity. In several *in vitro* studies, MeHg induced astrocyte swelling (Aschner 2000) and decreased transport of extracellular glutamate into the cell in cultures (Aschner 2000, Fonfria et al. 2005, Mutkus et al. 2005) or brain homogenate (Mutkus et al. 2005). Increased release of glutamate into the synapse has also been demonstrated with *in vitro* (Aschner 2000, Bondy et al. 1979, Fonfria et al. 2005) and *in vivo* MeHg treatment (Farina et al. 2003). As a result of decreased uptake and increased release of glutamate, concentrations in the synapse rise and may result in excitotoxicity (Albrecht et al. 1996). Perhaps to compensate for increased glutamate, levels of NMDA receptors decreased with increasing brain Hg in several species (Basu et al. 2007, 2009b, Scheuhammer et al. 2008). *In vitro*, MeHg inhibited binding to the NMDA receptor in neonatal and adult rats (Rajanna et al. 1997). Glutamatergic enzymes are also affected by Hg. Mercury inhibited glutamine synthetase activity in cultured astrocytes treated *in vitro* (Allen et al. 2001, Monnet-Tschudi et al. 1996) and cellular extracts treated immediately before assay (Allen et al. 2001, Engle et al. 1990, Monnet-Tschudi et al. 1996). However, effects of Hg *in vivo* may differ from those seen in vitro, as an elevation of GS has been found in rats exposed to MeHg (Kung et al. 1989).

GABA signaling is also impaired by MeHg. *In vivo* treatment with MeHg decreased GABA uptake in the dorsal ganglia (Araki et al. 1981), cerebral cortex and

caudate putamen (O'Kusky et al. 1989) of rats. Binding to the $GABA_A$ receptor decreased in mink treated *in vivo* with MeHg (Basu et al. 2010). In rat cerebellum, chronic MeHg treatment increased the number of benzodiazapene, but not GABA, binding sites (Concas et al. 1983). Similarly, acute treatment with 10mg/kg MeHg increased diazepam binding to the benzodiazepine site in several rat brain regions (Corda et al. 1981). Although *in vivo* exposure to MeHg did not alter benzodiazepine site binding in some other *in vitro* (Komulainen et al. 1995) and *in vivo* studies (Komulainen et al. 1985), *in vitro* treatment with MeHg decreased the ability of GABA site activation to enhance benzodiazepine binding (Komulainen et al. 1995). *In vitro*, GAD activity decreased with Hg (organic or inorganic) treatment in mink (Basu et al. 2010) and rat (Monnet-Tschudi et al. 1996). While GAD was unaffected by oral MeHg in adult rats (Concas et al. 1983), postnatal treatment of newborn rats with MeHg decreased GAD activity in the occipital cortex, striatum (O'Kusky 1988), frontal cortex, and caudate putamen (O'Kusky et al. 1985). Decreases occurred both at the onset of and preceding obvious neurological deficiencies (O'Kusky et al. 1985, 1988).

1.2 Knowledge Gaps

Previous work has shown that *in vitro* in and *in vivo* Hg treatment interferes with several aspects of glutamatergic and GABAergic signaling in laboratory rodents and cultured cells. However, these studies may not accurately portray effects in wild birds, which are exposed to numerous contaminants and stressors. Only one avian study has been completed to date to show associations between Hg exposure and

neurochemical receptor levels. A few have made similar associations in other wild species, but the evidence is largely correlative and conclusions have been drawn from correlative studies. Additionally, the significance of these changes is unknown because no studies have assessed the relationship between neurochemical and behavioral changes. Several recent papers have demonstrated that ecologically relevant Hg exposure may impact avian reproduction and behavior, but the underlying mechanisms have not been elucidated. By expanding our knowledge of how Hg disrupts neurochemistry and linking neurochemical and behavioral changes, it will be possible to more accurately predict Hg's ecological impacts.

1.3 Objective

The objective of this work was to support the use of changes in GABAergic and glutamatergic enzymes and receptors as biomarkers of Hg's subclinical neurological effects in birds and to determine the behavioral significance of the changes. As few data exist regarding effects of Hg on avian neurochemistry, this project has expanded our current knowledge to include effects in several wild and laboratory avian species and will deepen our understanding of effects on excitotoxicity related endpoints. Furthermore, this study was the first to assess the relationships between Hg exposure and changes in both neurotransmitter biochemistry and behavior.

1.4 Hypothesis

Mercury (Hg) is a potent neurotoxic environmental contaminant of concern. As methylmercury (MeHg) biomagnifies primarily through the aquatic ecosystem, fish eating birds are known to bioaccumulate Hg and are at risk for a host of organismal and population level effects. In the past, neurological problems resulting from acute exposure of birds to high levels of Hg were clearly observed in Europe, North America, and Asia. Although exposure to such high, toxic levels of MeHg is now rare, fish eating birds are now chronically exposed to Hg in the environment at levels associated with changes in productivity and neurobehavior, which may potentially impact population health.

Mercury is extremely neurotoxic at high levels, with effects in birds including pathological brain lesions and severe neurobehavioral changes. Before this clinical damage occurs, more subtle changes in brain neurochemistry can be observed with exposure to lower levels of Hg. Mercury associated biochemical changes in receptors and enzymes that mediate GABA and glutamate neurotransmission have been shown in rodent studies, and recent work has identified associations between Hg exposure and GABA and glutamate receptors in some fish eating mammals and birds. Such changes may provide an early indication of Hg's subclinical neurological effects, since GABA and glutamate are the main inhibitory and excitatory neurotransmitters. However, wildlife work to date has been restricted to studying neurochemical receptors, and the value of these changes as biomarkers in avian risk assessment is limited because the work is largely descriptive and the significance of the changes to avian behavior are yet unknown.

The overall goal of this research was to explore for relationships between environmental Hg exposure and biochemical alterations in excitatory neurotransmission in birds, and to determine if these alterations warn of behavioral changes capable of affecting survival and reproduction in wild species. **The hypothesis is that exposure to environmentally relevant levels of Hg induces subclinical changes to key receptors and enzymes of GABAergic and glutamatergic neurotransmission in fish eating birds which results in alterations in ecologically relevant behaviors, and that these changes can be used as biomarkers to improve understanding of Hg exposure and effects.** This hypothesis was tested by collecting evidence from relevant field exposure studies on multiple species, *in vivo* whole animal egg injection studies in the laboratory, and *in vitro* studies on cultured neurons.

1.5 Specific Aims

The overall hypothesis was investigated through the following specific aims:

<u>AIM 1: Wildlife studies</u>

Characterize associations between environmental exposure to Hg (as assessed by brain Hg analysis) and alterations in neurochemical receptors (NMDA, $GABA_A$ (benzodiazepine)) and enzymes (glutamine synthetase, glutamic acid decarboxylase) in brain tissues of several wild caught, fish eating birds.

<u>AIM 1.1: Bald eagle study</u>

Assess relationships between neurochemical receptors and enzymes and Hg exposure in wild collected bald eagles from across US Great Lakes states.

AIM 1.2: Herring gull study

Assess relationships between the NMDA, muscarinic cholinergic, and nicotinic cholinergic receptors and total brain Hg in the brains of adult herring gulls captured from colonies across the Great Lakes.

AIM 2: Laboratory neurochemistry studies

Use controlled *in vivo* (injection of fertile eggs with Hg) laboratory studies to support a causal relationship for the associations explored in Specific Aim 1.

AIM 2.1: White leghorn chicken studies

Assess relationships between mercury exposure and changes in GABAergic and glutamatergic receptors and enzymes in white leghorn chicken embryos exposed *in ovo* via egg injection (commercially purchased eggs).

AIM 2.2: Thick-billed murre study

Assess relationships between mercury exposure and changes in GABAergic and glutamatergic receptors and enzymes in thick billed murre embryos exposed *in ovo* via egg injection (wild collected eggs).

AIM 3: Laboratory behavior studies

Determine in laboratory chickens if environmentally relevant levels of Hg are capable of causing changes in behaviors or motor skills that might affect survival or reproduction.

AIM 4: Postmortem stability studies

Support the use of neurochemical receptors (NMDA, $GABA_A$ (benzodiazepine)) and enzymes (glutamine synthetase, glutamic acid decarboxylase) as biomarkers for use in field studies by demonstrating sufficient post mortem stability.

AIM 4.1: Literature review

Review human and rodent literature to demonstrate that the receptors and enzymes are relatively stable post mortem and may be used in field based ecological studies.

AIM 4.2: Postmortem stability of chicken brain under environmental conditions

Perform a laboratory study designed to mimic field conditions to demonstrate in chicken brain that the receptors and enzymes are relatively stable post mortem and may be used in field based ecological studies.

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Chapter 2

Investigation of spatial trends and neurochemical impacts of mercury in herring gulls across the Laurentian Great Lakes[1]

2.1. Introduction

Fish-eating birds bioaccumulate appreciable levels of mercury (Hg), especially methylmercury (MeHg) from their environment (Scheuhammer et al., 2007; Wolfe et al., 1998). This is of concern because MeHg is a potent neurotoxicant (Clarkson and Magos, 2006). In captive American kestrels (*Falco sparverius*), MeHg altered parental incubation behavior (Albers et al., 2007) and impaired coordination to a degree that might have interfered with effective reproduction resulting in infertile eggs (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). In wild common loons (*Gavia immer*), MeHg exposure caused behavioral alterations that detrimentally affected reproductive capacity and parent-chick relationships (Evers et al., 2008; Nocera and Taylor, 1998). Collectively, these studies show that MeHg can impair avian neurobehavior which may ultimately affect population health. It is important to resolve the early mechanisms by which Hg disrupts reproduction and brain function, as avian productivity is reduced in environments

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high in MeHg (Burgess and Meyer, 2008). Before causing overt neurological damage, MeHg will first cause reversible and subtle changes to neurochemistry (Manzo et al., 1996). A recent study of brain tissue from two fish-eating birds, the bald eagle (*Haliaeetus leucocephalus*) and common loon, found that Hg concentration was positively associated with muscarinic cholinergic receptor levels and negatively associated with N-methyl-D-aspartic acid (NMDA) receptor levels (Scheuhammer et al., 2008). These two receptors have important roles in animal cognition, motor function, and reproduction (Ozawa et al., 1998; Wess, 2004). Similar Hg-associated neurochemical effects have been observed in mammalian wildlife, marine mammals, and captive animals exposed to MeHg (Basu et al., 2005a, 2005b, 2006a, 2007a, 2007b, 2007c, 2009). Neurochemical receptors can be used as biomarkers to detect potentially reversible and subclinical changes that precede irreversible damage to the function and structure of the brain (Manzo et al., 1996).

Herring gulls are fish-eating birds that bioaccumulate environmental contaminants. They are colonial breeders, year-round residents on the Great Lakes, and are numerous and widely distributed throughout the Great Lakes (Fox, 2001). For these reasons, herring gulls have been used by Environment Canada's Canadian Wildlife Service for more than 30 years to monitor levels, trends, and effects of contaminants in the Great Lakes region (Mineau et al., 1984; Peakall and Fox, 1987, Koster et al., 1996). As part of this monitoring program, Hg levels have been measured in herring gull eggs. Over a 20 year period from 1972-1992, egg Hg levels were variable across colonies but displayed a decreasing trend that appeared to

slow or stabilize in the 1980s and 1990s. Mean values at one colony during this period reached 0.88 μg/g wet weight (Koster et al., 1996).

Though herring gulls are exposed to Hg, levels in the brain and the potential neurotoxic effects have not yet been explored. The objectives of the present study were to: (1) report on total Hg levels in the brains of herring gulls; (2) determine if brain Hg levels vary across geographical sites; and (3) determine if brain Hg levels can be related to changes in the levels of muscarinic and nicotinic cholinergic receptors and NMDA receptors as determined via biochemical (i.e., [3H]-ligand binding assays) and molecular (i.e., mRNA expression of the nicotinic receptor, nAChR α-7) methods.

2.2. Methods

2.2.1 Animals and Study Area

Breeding adult herring gulls were trapped on their nests using box traps during mid-incubation at five Great Lakes colonies during the springs of 2001 and 2003; Hamilton Harbour (HH) in western Lake Ontario (2003); Scotch Bonnet Island (SBI) in eastern Lake Ontario (2003); Chantry Island (CH) in eastern Lake Huron (2001); Middle Sister Island (MSI) in western Lake Erie (2001); and Fighting Island (FGT) in the Detroit River (2001). Additionally, gulls were trapped at Kent Island (KNT), a non-Great Lakes reference colony in the Bay of Fundy (2001). Gulls were euthanized by decapitation using a small animal decapitator and brain tissues were excised and immediately frozen in liquid nitrogen. In the laboratory, cerebral

hemispheres were dissected and preserved optimally for neurochemical receptor binding assays, gene expression studies, and chemical (e.g., Hg) analyses.

2.2.2 Hg Analysis

Brain tissues for Hg analysis were dried at 60°C for 48 hours. Total Hg (THg) concentrations were measured in these dried tissues as described previously (Scheuhammer et al., 2008) using a Direct Mercury Analyzer (DMA-80, Milestone Inc., CT). Quantification of THg using a DMA is recognized by the U.S. EPA (Method 7473). All residue information is reported on a dry weight basis.

For quality control, sample blanks, intermittent duplicate samples, and Standard Reference Materials (TORT-2 and DOLT-3, Analytical Chemistry Unit, National Research Council of Canada) were included in all batch runs. Average accuracy was within 10% of certified values for all analyses and analytical precision (% relative standard deviation of replicate samples) averaged <10% for all analyses.

2.2.3 [3H]-Ligand Binding to Neurochemical Receptors

For receptor ligand binding assays, cellular membranes were prepared from brain tissues using protocols described elsewhere (Basu et al., 2005a, 2006b). Binding to the muscarinic (mACh) and nicotinic nACh) (Na/K buffer: 50 mM NaH_2PO_4, 5 mM KCl, 120 mM NaCl, pH 7.4) and NMDA (Tris buffer: 50 mM Tris, 100 μM glycine, 100 μM L-glutamic acid, pH 7.4) receptors was performed in the buffers indicated. Thirty μg of membrane preparation was re-suspended in buffer and added to microplate wells containing a 1.0 μM GF/B glass filter (Millipore, Boston, MA, USA). For muscarinic and nicotinic receptor binding, samples were incubated

with 1 nM [^{3}H]-QNB (42 Ci/mmol; NEN/Perkin Elmer, Boston, MA, USA) and 1 nM [^{3}H]-Cytisine (26.8 C/mmol; NEN/Perkin Elmer), respectively, for 60 min. For NMDA receptor binding, samples were incubated with 5 nM [^{3}H]-MK-801 (22 Ci/mmol; NEN/Perkin Elmer) for 120 min. All assays were carried out with gentle shaking at room temperature and binding reactions were terminated by vacuum filtration. The filters were rinsed three times with buffer and then allowed to soak for 96 hrs 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 and nACh 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.

2.2.4 Expression of nAChR α-7 mRNA

TRIzol reagent (Invitrogen, Burlington, Ontario) was used to isolate total RNA from cortex tissue (~100 mg) of all individuals (n=8-10/site). Total RNA (1.5 µg) was annealed with 150 ng random primers (Invitrogen) and complimentary DNA (cDNA) was generated using Superscript II RNase H-reverse transcriptase (Invitrogen). A previously described real-time reverse transcription PCR (real-time

RT-PCR) assay (Crump et al., 2008) was used to determine the relative mRNA expression levels of herring gull nAChR α-7 (GenBank Accession #: AY914169). Briefly, the Brilliant QPCR Core Reagent kit (Stratagene, La Jolla, CA) was used for the multiplex assay; nAChR α-7 in duplex with the contol gene, β-actin. Standard curves were generated for both genes from serial dilutions of cDNA and MxPro v3.00 software (Stratagene) was used to calculate the relative quantities of each gene. Relative quantity values were normalized to the internal control gene, β-actin.

2.2.5 Statistical Analyses

The critical level of significance was set at $\alpha = 0.05$. Mercury levels were log10 transformed to achieve a normal distribution and equal variance (determined by Levene's test). Following back-transformation, THg results are reported as mean ± standard deviations. Levels of brain Hg and neurochemical receptor binding across the six study sites were compared with one-way ANOVAs (Tukey's post hoc). Because muscarinic receptor data did not meet the assumption of equal variance, a Kruskal-Wallis test rather than ANOVA was used for comparison of this receptor between sites. By pooling all data, the association between brain THg and the neurochemical receptor levels and nAChR α-7 mRNA expression was also determined by Pearson's correlation. Quartile analysis was also performed on neurochemical receptor level data. All statistical operations were performed with SPSS (Version 11.5, SPSS Inc., Chicago, IL, USA).

2.3 Results

THg in herring gull brain tissue ranged from 0.14 μg/g to 2.01 μg/g with an overall mean of 0.54 μg/g. THg levels varied significantly among sites (ANOVA $p<0.0001$), with levels at SBI significantly higher than all other sites (Tukey $p<0.001$) (Fig. 2.1; Table 2.1). Mean levels at CH were significantly lower than at SBI, MSI, and FGT ($p\leq0.01$) and marginally significantly lower than at HH ($p=0.054$).

Levels of neurochemical receptors varied between some sites (ANOVA $p<0.05$) (Table 2.1). For example, mean NMDA receptor levels at CH differed significantly from KNT ($p<0.05$), and mean nicotinic levels at CH differed significantly from KNT and MSI ($p<0.05$). For correlation assessment, neurochemical parameters were plotted against log transformed values of THg. Correlations were not significant between THg and the mAChR ($r = -0.22$, $p=0.10$), nAChR ($r =-0.19$, $p=0.16$), or NMDA receptor ($r=-0.22$, $p=0.10$) (Fig. 2.2). Additionally, all birds were subdivided into quartiles based on brain THg levels. The lower quartile for THg was 0.33 μg/g, the median was 0.44 μg/g, and the upper quartile was 0.62 μg/g. When compared using ANOVA, no differences were found between quartiles for muscarinic ($p=0.208$), nicotinic, ($p=0.234$), or NMDA receptor levels ($p=0.407$).

The cycle threshold (Ct) values for the internal control gene, β-actin, were stable across all individuals and the no-template negative controls did not amplify. mRNA levels of nAChR α-7 did not vary by more than 1.5 fold within or among the six colonies. Interindividual variation was minimal and no significant correlation

was observed between total brain Hg levels and mRNA expression of nAChR α-7 ($r=0.022$, $p=0.872$).

2.4 Discussion

Herring gulls accumulate the neurotoxic metal Hg but concentrations in their brains have not previously been reported. Mercury levels found in this study (0.1-2.0 μg/g) were lower than those recently measured in the brains of common loons (0.2-68 μg/g) and bald eagles (0.3-23 μg/g) (Scheuhammer et al., 2008) (Table 2.2). Levels of Hg measured in the brains of North American adult black crowned night herons, snowy egrets (Henny et al., 2002), double crested cormorants (Henny et al., 2002; Sepulveda et al. 1998), ospreys (DesGranges et al. 1998), and western grebes (Elbert and Anderson, 1998) were also several times higher than in herring gulls (Table 2.2). While many of these other birds were sampled from contaminated sites, even those sampled from a clean reference site had higher levels than the gulls studied here. Interestingly, brain THg levels in herring gulls were comparable to those in Franklin gulls (Burger and Gochfeld, 1999) (Table 2.2), which are also members of the family *Laridae*. The lower THg concentration in the gulls compared to the other fish-eating birds may be attributed to dietary differences. Gulls are considered to be opportunistic fish-eaters and during the breeding season they feed mainly on alewife and rainbow smelt (Fox et al., 1990; Hebert et al., 1999), which are low trophic organisms. Their diet is also supplemented by available garbage and locally abundant nonfish food sources like insects and plants that likely are not high in Hg (Fox et al., 1990; Ewins et al., 1994). Considering that fish, especially

those that feed at higher trophic levels, are the main source of Hg exposure in obligate fish-eaters, it is unsurprising that gulls carry lower burdens of THg in their brains than some other fish-eating species.

No Hg-associated changes in neurochemical receptor levels were found in herring gull brains. Though a precise threshold value for Hg-associated neurochemical effects has yet to be determined for wildlife, the mean levels of THg in the herring gull brain were 2-13 times lower than mean values in some other fish-eating birds for which brain THg concentrations have been reported (Table 2.2). Levels were 6-9 times lower than brain Hg levels measured in avian species (common loons and bald eagles) where Hg-associated neurochemical changes have been observed (Scheuhammer et al., 2008). Similarly in mammals, brain Hg levels in fish-eating wild mink and river otters where Hg-associated neurochemical changes have been reported are much greater than levels measured here in herring gulls (Basu et al., 2005a, 2005b, 2006a, 2007a, 2007b, 2007c). In addition to employing biochemical methods to study neurochemical receptor levels, we used molecular approaches and explored mRNA expression of the nicotinic receptor, nAChR α-7. This was permissible since brain tissues were excised immediately from animals in the field and flash frozen in liquid nitrogen. Similar to what we found when using 3H-ligand binding techniques, nicotinic receptor mRNA expression was not associated with brain THg levels. Owing to the lack of significant relationships between brain Hg and neurochemical biomarker results, we did not expand our molecular investigation to include mRNA expression studies on other receptors.

Although THg levels in herring gull brains were previously unknown, studies have reported Hg values from eggs and kidneys from Great Lakes herring gulls, including animals from the sites used in the current study. Consistent with the findings of previous studies, Hg levels in the current study, with the exception of SBI, did not vary significantly among sites. Mercury levels in herring gull eggs from Great Lakes sampling sites have, in general, shown significant declines over the period from 1972-1992, but were generally highest at Lake Ontario sites including SBI and the nearby Little Galloo and Snake Islands. Levels at these eastern Lake Ontario islands were often 2-3 fold higher than at other sites (Koster et al., 1996). A survey of environmental contaminants in herring gull eggs from 1998-2002 reported no differences in Hg among Great Lakes sites, including an eastern Lake Ontario (Snake Island) site close to SBI (Weseloh et al., 2006); however, eggs may not be the best indicator of Hg levels in birds at a site, as egg concentrations do not always correlate with tissue concentrations at the site (Fox et al., 2002; Dauwe et al., 2005). A tissue based study by Fox et al. (2002) in the early 1990s showed little overall variability in renal Hg levels in adult gulls at 16 Great Lakes sites, but as in the current study, renal Hg levels at SBI exceeded those at other sites approximately 2-3 fold. Stable isotope data (δ15N values) indicate that herring gulls at SBI feed at a high trophic level relative to birds at other sites (Craig Hebert, Environment Canada, unpublished data), therefore emphasizing the importance of diet for explaining the variability in Hg exposure and tissue accumulation. It should also be noted that fatty acid signatures and carbon and nitrogen isotopes in herring gulls sampled from 1978-2005 have indicated a decline in fish consumption and an

increase in terrestrial food consumption as aquatic prey abundance in the Great Lakes has decreased (Hebert et al., 2008). This finding suggests that herring gulls may be exposed to lower levels of contaminants, such as Hg, that are derived from fish consumption. Higher Hg levels in gulls from SBI may also be realized by considering that atmospheric deposition of Hg across the North American landscape generally increases from west to east. The colony at SBI was the most easterly freshwater colony investigated here and it may experience heightened Hg deposition (and thus higher brain THg levels) when compared to other herring gull colonies within the Great Lakes basin. A continent-wide geographical trend has previously been reported in a study of common loons across North America where highest levels of blood and feather Hg were found in birds residing in the east (Evers et al., 1998). Though, in this study on loons the authors noted that in the Upper Great Lakes (Minnesota, Wisconsin, and Michigan's Upper Peninsula) that variation in blood and feather Hg was likely more influenced by biogeochemical factors than by atmospheric deposition.

2.5 Conclusions

In summary, this study increases our understanding of spatial trends of mercury pollution in the Great Lakes region and examines linkages between mercury exposure and neurochemical biomarkers in a key sentinel species. When coupled with other studies conducted on a wide regional scale, this work on herring gulls suggests that wildlife in the eastern region of the Laurentian Great Lakes basin may experience higher accumulations of Hg. Though, it should be emphasized that

spatial trends in Hg accumulation can vary tremendously and are influenced by factors such as atmospheric deposition loads, regional biogeochemistry, and an organism's trophic ecology.   Several studies have recently documented that ecologically relevant Hg levels may cause neurological and behavioral impacts in fish-eating birds and thus a second aim of this work was to determine whether Hg was of neurological concern to the herring gull. By considering Hg levels in the herring gull brain and associating these levels with several neurochemical biomarkers previously shown to be impacted by Hg, this work suggests that Hg may not be of neurological concern to the herring gull.

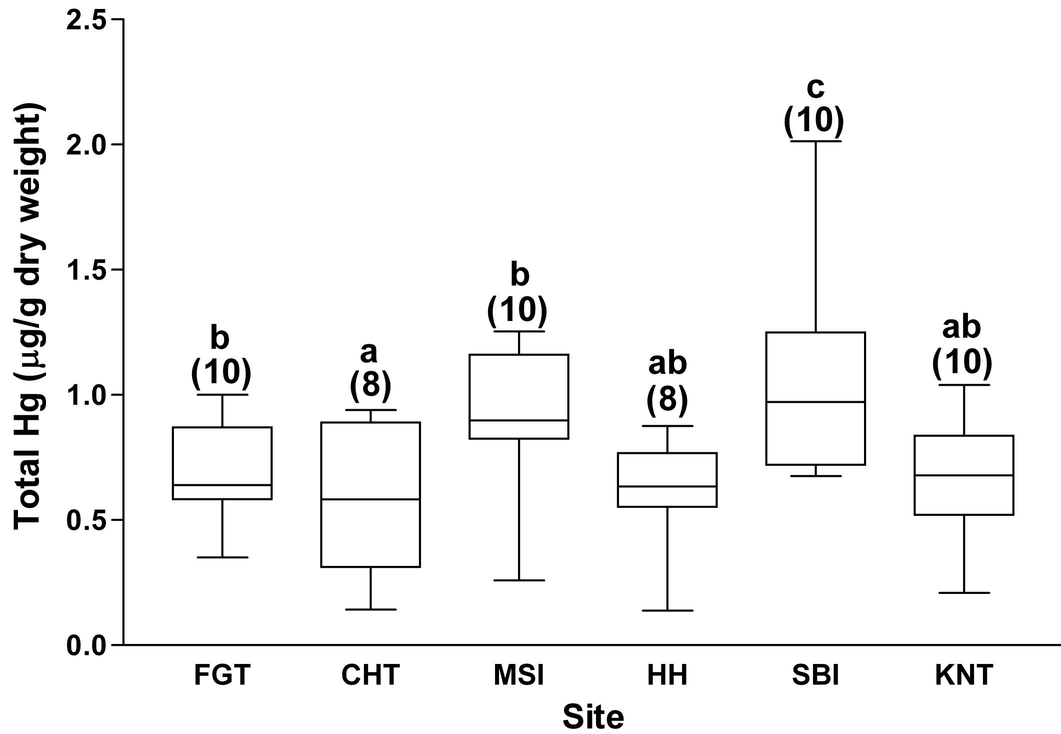

Figure 2.1 Total mercury (THg) levels in the brains of adult herring gulls collected from five Great Lakes colonies and one non-Great Lakes reference colony. Boxes represent upper/lower 25th percentiles, horizontal lines represent median, and vertical lines represent maximum/minimum observations. Letters indicate site mean significantly different from other colonies ($p<0.001$). Sample sizes are in parentheses

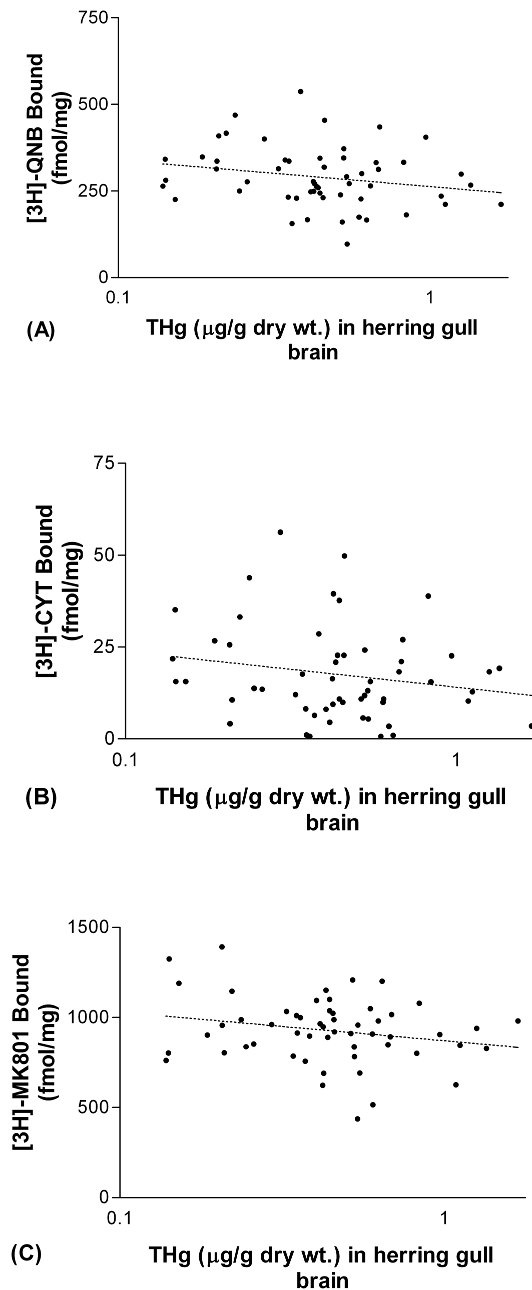

Figure 2.2 Mercury and nerochemical receptor levels in herring gulls. Associations between total mercury (THg) and neurochemical receptors in brains of adult herring gulls (n=55) collected from five Great Lakes colonies and one non-Great Lakes reference colony. (A: [3H]-QNB binding to muscarinic cholinergic receptor; B: [3H]-CYT binding to nicotinic cholinergic receptor; and C: [3H]-MK801 binding to NMDA receptor). Dashed lines indicate best fit linear regression

Table 2.1 Brain mercury and neurochemical receptor levels and expression in herring gulls. Mean (± standard deviation) total mercury (THg; µg/g dry weight), neurochemical receptor levels (fmol/mg; as determined by [3H]-QNB binding to mAChR, [3H]-CYT binding to nAChR, and [3H]-MK801 binding to NMDA receptor), and relative mRNA expression of nAChR α-7 in the brain of herring gulls at five Great Lakes colonies and one non-Great Lakes reference colony. Letters denote significant differences (Tukey p<0.05) for a given column.. Sample sizes are indicated in parenthesis

Site	THg	mACh Receptor Binding	nACh Receptor Binding	NMDA Receptor Binding	Relative nAChR α-7 mRNA expression
Chantry Island	0.25±0.18 (8)	345.74 ± 71.33 (8)	29.41 ± 12.94[b] (8)	1092.36 ± 206.63[b] (8)	0.91 ± 12 (8)
Fighting Island	0.48±0.17 (10)	279.77 ± 135.60 (10)	15.55 ± 13.34[ab] (10)	958.76 ± 144.85[ab] (10)	0.86 ± 24 (10)
Hamilton Harbour	0.45±0.19 (8)	319.05 ± 69.07 (8)	24.22 ± 13.57[ab] (8)	908.97 ± 137.58[ab] (8)	0.82 ± 16 (8)
Kent Island	0.38±0.13 (8)	281.33 ± 67.61 (10)	12.77 ± 10.43[a] (10)	830.76 ± 199.77[a] (10)	0.95 ± 18 (10)
Middle Sister Island	0.49±0.15 (10)	277.84 ± 35.46 (10)	12.15 ± 7.09[a] (10)	887.27 ± 177.76[ab] (10)	1.38 ± 34 (10)
Scotch Bonnet Island	1.11±0.5[a] (10)	249.66 ± 92.34 (9)	14.53 ± 11.48[ab] (9)	915.09 ± 138.88[ab] (9)	0.90 ± 14 (10)

Table 2.2 Mean values (and range) of total mercury (THg) in the brains of fish-eating birds. For values provided on a wet weight basis, concentrations were converted to a dry weight basis assuming 80% moisture content (indicated by letter superscript)

Species	Location	THg (μg/g)	Study
bald eagle	Canada	3.0 (0.3-23)	Scheuhammer et al., 2008
common loon	Canada	4.6 (0.2-68)	Scheuhammer et al., 2008
black crowned night heron	Nevada (reference /contaminated)	2.5 (0.33-3.8)/8.7 (4.2-27.55)	[a] Henny et al., 2002
snowy egret	Nevada (reference /contaminated	4.5 (2.8-7.55)/11.5 (9.75-19.2)	[a] Henny et al., 2002
double crested comorant	Nevada (reference /contaminated)	6.1 (4.75-7.2)/56.3 (44.45-74.5)	[a] Henny et al., 2002
double crested cormorant	Florida	7.5 (0.4-42)	[a] Sepulveda et al., 1998
osprey	Canada (reference /contaminated)	1.15 (0.4-2.0)/5.05 (1.05-9.45)	[a] DesGranges et al., 1998
western grebe	California	1.1 (range not available)	[a] Elbert and Anderson, 1998
Franklin's gull	Minnesota	0.23 (range not available)	[a] Burger and Gochfeld, 1999
herring gull	Great Lakes	0.54 (0.1386-2.0135)	Current study

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Chapter 3

Mercury exposure and neurochemical impacts in bald eagles across several Great Lakes states[2]

3.1 Introduction

Bald eagles (*Haliaeetus leucocephalus*), like other long-lived piscivorous birds, bioaccumulate mercury (Hg). Numerous studies on bald eagles across North America have documented appreciable Hg levels in eggs (Weimeyer et al. 1993), feathers (Bowerman et al. 1994; Wood et al. 1996; Weech et al. 2006; Bechard et al. 2009), blood (Wood et al. 1996; Weech et al. 2006), liver (Wood et al. 1996; Weech et al. 2003; Scheuhammer et al. 2008), and brain (Scheuhammer et al. 2008). Though Hg exposure in bald eagles is fairly well understood, much less is known about adverse health effects that may be associated with such exposures.

It is well established that Hg is neurotoxic. Methylmercury (MeHg) in fish readily crosses the blood-brain barrier and, due to Hg's affinity for protein thiols, may target and disrupt receptors, enzymes, and other cellular components of the brain (Clarkson et al. 2006). Pathologically, exposure to high Hg levels is associated with demyelination, neuronal degeneration, and inflammation in birds (Spalding et al. 2000). Perhaps due to underlying changes to the brain, Hg exposure has been linked to impaired motor skills and cognitive behaviors in species including

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common loons (Evers et al. 2008; Kenow et al. 2010), American kestrels (Bennett et al. 2007), and great egrets (Spalding et al. 2000). Such neurobehavioral changes potentially affect survival and reproduction. Mercury is associated with decreased reproductive success in the laboratory (e.g. American kestrels; Albers et al. 2007) and field (e.g. common loons; Burgess et al. 2008; Evers et al. 2008). Because impacts on survival and reproduction may affect both individual and population health, it is important to develop markers that may help recognize early, subclinical effects of Hg on the brain.

Alterations in neurochemical receptors and enzymes relate to brain function and indicate neurological damage before structural and functional toxicity are apparent (Manzo et al. 1996). Recently, several studies in fish-eating wildlife have identified associations between Hg and neurochemical biomarkers involved in various neurotransmitter systems. Species studied include mink (Basu et al. 2007b), river otters (Basu et al. 2007a), polar bears (Basu et al. 2009a), common loons, and bald eagles (Scheuhammer et al. 2008). Comparable effects have been seen *in vitro* and in animals exposed to MeHg in laboratory studies (Basu et al. 2005, 2006, 2007b). Collectively, these studies demonstrate that Hg exposure may have subtle neurological impacts on many species.

Receptors and enzymes that mediate glutamate and γ-aminobutyric acid (GABA), the primary excitatory and inhibitory neurotransmitters, are tightly regulated and disruption can result in excitotoxicity (Reis et al. 2009). Rodent studies show that Hg alters components of both pathways (Concas et al. 1983; Rajanna et al. 1997; Monnet-Tschudi et al. 1998), but these effects are not well

documented in birds. Because glutamate and GABA play roles in learning and coordination (Scholes 1965; Gibbs et al. 2008), changes in components of these systems may disrupt ecologically important behaviors.

To date, much of the work on Hg in eagles has been limited to monitoring studies assessing levels in feathers or blood. A recent study demonstrated associations between brain Hg levels and neurochemical receptors (N-methyl-D-aspartic acid (NMDA), muscarinic cholinergic) in bald eagles from Canadian provinces (Scheuhammer et al. 2008), thus suggesting that Hg may be of neurological concern to this species. Although Hg is of concern to Great Lakes ecosystem health, levels in the brain and related neurological effects in eagles in the region are unknown. The first goal of this study was to assess Hg exposure (total (THg), organic (OHg), and inorganic (IHg)), through measurements in brain, liver, and feathers, in bald eagles collected from five U.S. states in the Great Lakes region (IA, OH, MI, MN, and WI) from 2002-2010. The second goal was to determine if brain Hg levels were associated with changes in neurochemical receptors (NMDA and $GABA_A$ (benzodiazepine)) and enzymes (glutamic acid decarboxylase (GAD), glutamine synthetase (GS)) involved in glutamate and GABA neurotransmission, most of which have not been investigated in wildlife toxicology studies.

3.2 Methods

3.2.1 Animals

Carcasses (n=135) were provided by Michigan Department of Natural Resources and Environment, Ohio Department of Natural Resources, Saving our

Avian Resources (Iowa), Wisconsin Department of Natural Resources, The Raptor Center (University of Minnesota), and Minnesota Department of Natural Resources. Birds died of various causes in the field or during rehabilitation. Carcasses were collected between 2002 and 2010 and stored at -20°C until tissue (liver, whole brain, breast feather (whole), and primary feather (distal 2cm)) collection. Samples from all age classes were studied. Of birds for which sex data were available, 35% were male and 65% were female. Extracted tissues were stored at -80°C until all analyses. Proper USFWS permits were obtained for this work.

3.2.2 Hg Analysis

Total and organic Hg were analyzed using methods described previously (Nam et al. 2010). Prior to analysis, brain and liver tissues were dried at 60°C for 48 h. and crushed into a fine powder. Feathers were washed three times in acetone then deionized water, and were minced using stainless steel scissors and dried at room temperature for 24 h. OHg was extracted from dried liver and brain using micro-scale organic extraction methods described elsewhere (Nam et al. 2010). THg and OHg were measured by thermal decomposition, amalgamation, and atomic absorption spectrophotometry (Basu et al. 2009a) in a Direct Mercury Analyzer (DMA-80, Milestone Inc., CT). Intermittent blanks, duplicate samples and standard reference materials (SRMs) (TORT-2, DOLT-3, and DOLT-4, National Research Council of Canada) were included to monitor accuracy and precision. For THg, average accuracy was 98% of certified values and average precision (% relative standard deviation) was 5%. For OHg, average accuracy was 104% of certified

values and average precision was 20%. Residue information is reported on a dry weight basis. Inorganic Hg was calculated by subtracting OHg from THg. Detection limits for THg ranged from 0.03-1.04 ng and detection limits for OHg ranged from 0.03-2.09 ng.

3.2.3 Se Analysis

Selenium (Se) was analyzed in liver and brain from a subsample of 46 eagles. Selenium was detected using an Inductively Coupled Plasma Mass Spectrometer (ICPMS; Agilent 7500c, Agilent Technologies, Palo Alto, CA) equipped with a quadrupole analyzer and octopole collision/reaction cell pressurized with hydrogen or helium reaction gas. All samples were digested overnight in 70% nitric acid (as 2% nitric acid solution) in a single batch run. 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 ICPMS was tuned using a solution of 10ppb of Li, Y, Ce, Tl, and Co (Agilent internal standard mix). 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. Residue information is reported on a dry weight basis. The limit of detection, determined as the mean of blanks plus 3 times the standard deviation of the mean,

was 0.024 ng/g. Accuracy and precision from SRMs (DOLT-4) was within 12% of expected values.

3.2.4 Neurochemical Assays

Cellular homogenates, enzyme supernatants, and membranes were prepared according to modifications of a procedure described elsewhere (Basu et al. 2006). Brains were homogenized in 50 mM Tris buffer (50 mM Tris HCl, 50 mM Tris Base, pH 7.4). Supernatant was collected after centrifuging at 15000 x g for 15 min., and membranes were isolated by centrifuging at 48000 x g for 15 min., washing three times, and resuspending in Tris buffer. The Bradford assay was used to determine protein content. Prepared samples were stored at -80°C.

GS activity was measured in supernatant based on the assay described by Santoro et al. (2001) with changes described below. Hydroxylamine hydrochloride and imidazole concentrations were increased to 50 mM and 100 mM, and sodium arsenate was used in place of arsenic acid. Assays were performed on supernatant (400 μg/ml) in 1.7 ml microcentrifuge tubes and incubation was in a 37°C shaking heating block. Absorbance was read at 500nm on a Nanodrop ND-1000 spectrophotometer (Thermo Scientific) and values were compared to a standard curve to determine glutamyl-γ-hydroxymate concentration and specific activity. Samples were assayed in triplicate and a pooled control sample was used to monitor variability. Between day variability averaged 10% and within day variability averaged 7%.

GAD activity was measured in brain homogenate according to a modification of previously described methods (Basu et al. 2009b). The reaction mixture was added to a 75mm glass tube containing 50 μl (100 μg) of homogenate. This tube was placed in a 100mm glass tube and a Whatman GF/A filter soaked in 100 μl Scintigest (Fisher Scientific, USA) was placed between the tubes. The reaction was started with the addition of 10 mM glutamate containing 0.3 μCi l-[U-^{14}C] glutamic acid (260 mCi/mmol; Perkin Elmer, USA) and the tube was sealed and incubated for 45 min. in a 37°C shaking heating block. The reaction was terminated with injection of 0.5 ml of 0.25 M HCl and tubes were incubated for an additional 60 min. After soaking in OptiPhase Supermix Cocktail (Perkin Elmer) for 24 h., radioactivity on the filters was measured on a liquid scintillation counter (Wallac 1450 Microbeta Plus, Perkin Elmer). Samples were assayed in duplicate and a pooled control sample was used to monitor variability. Between-day variability averaged 17% and within day variability averaged 13%.

Receptor binding assays were performed using cellular membranes according to previously published methods (Basu et al. 2009a). For both receptors, protein (30 μg) was suspended in 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 2h. (NMDA) or on ice with 2 nM [3H]-flunitrazepam (81.4 Ci/mmol) for 30 min. (GABA). Nonspecific binding was determined through coincubation with 100 μM unlabeled MK-801 (NMDA) or 20 μM clonazepam (GABA). Samples were

assayed in quadruplicate and variability, which averaged 15%, was monitored through internal, pooled controls. Binding is reported as fmol of radioisotope bound per mg of membrane protein (fmol/mg).

3.2.5 Statistical Analyses

Statistical analyses were performed using PASW Statistics (V.17.0, Chicago, IL, USA) and Stata (V.11) (for quantile regression). A P-value of <0.05 was considered significant. Hg and Se data were log transformed prior to analyses to achieve a normal distribution, and were back transformed for reporting. THg levels were compared across states using one-way ANOVA (Tukey's post hoc). Correlations (Pearson or Spearman) were used to determine relationships between Hg concentrations in different tissues and between Hg or Se concentrations and neurochemical endpoints. Simple linear regression was used to determine equations when appropriate. Quantile regression was performed to investigate Hg as a limiting factor for GAD activity (Cade et al. 1999). ANOVA and t-tests were used to compare neurochemical endpoints between age and gender categories.

3.3 Results

3.3.1 Brain Hg and Se

An overview of THg concentrations in brain is provided in Table 3.1. In birds for which sex information was available, concentrations did not differ significantly between sexes. Mean concentrations of brain THg increased with age group and differences were detected between groups (Table 3.1). Brain THg varied

significantly among the states (Fig. 3.1). On average, 64.1% (range:2.8%-98.0%) of THg in the brain was organic. Generally, demethylation increased as THg in the brain increased.

Levels of Se in the brain averaged 3.24 μg/g ± 2.54 μg/g (range:1.17 -17.92). Brain Se was positively correlated with brain THg ($p<0.001$, $r_p=0.732$), OHg ($p=0.003$, $r_p=0.434$), and IHg ($p<0.001$, $r_p=0.685$). The mean molar ratio of THg:Se was 0.38 ± 0.26 (range:0.06 -1.24). The molar concentration of THg surpassed that of Se in the brain of only one bird. The molar ratio of THg:Se correlated positively with THg ($p<0.001$, $r_s=0.957$), OHg ($p<0.001$, $r_s=0.670$, and IHg ($p<0.001$, $r_s=0.810$).

3.3.2 Liver Hg and Se

THg concentrations in the liver (Table 3.1) were higher than those in the brain. Concentrations did not differ significantly among states (Fig. 3.1). THg concentrations in liver were positively correlated with those in brain ($p<0.001$, $r_p=0.805$, logBrainTHg=-0.197 + 0.684 logLiverTHg). The average percent of THg in the organic form was 59.33% (range:11%-107%) and as in brain demethylation increased with THg concentrations . Se concentrations in liver averaged 6.07 μg/g ± 3.47 μg/g (range:1.33 -16.9). As in brain, Se was positively correlated with THg ($p<0.001$, $r_p=0.774$), OHg ($p=0.003$, $r_p=0.434$), and IHg ($p<0.001$, $r_p=0.685$) in liver. Unlike the brain, in the liver there was less of an excess of Se over Hg and the molar ratio of THg:Se approached or exceeded 1 in several birds. The mean molar ratio of

THg:Se in the liver was 0.50 ± 0.42 (range:0.5 -2.4). The ratio was positively correlated with liver THg levels (p<0.001, r_s=0.912).

3.3.3 Feather Hg

THg in breast feathers averaged 15.84 µg/g ± 13.49 µg/g (range:0.54-75.05; n=106). THg in primary feathers averaged 15.31 µg/g ± 10.63 µg/g (range:1.73 - 15.31; n=50). THg in breast feathers correlated with THg in primary feathers (p<0.001, r_s=0.617), liver (p<0.001, r_p=0.610), and brain (p<0.001, r_p=0.611, logBrainTHg=-0.357 + 0.630 logBreastFeatherThg). Though THg in primary feathers increased with THg in brain and liver, these relationships were not statistically significant.

3.3.4 Neurochemical Assays

To assess relationships between Hg and neurochemical biomarkers, receptor levels and enzyme activity were plotted against log transformed THg, OHg, and IHg. Brain Thg was associated with an increase in GS activity (p=0.028, r_p=0.190) (Fig. 3.2a). When OHg and IHg were examined separately, the relationship with GS was significant for IHg (p=0.030, r_p=0.188) but not OHg. GAD activity was not significantly associated with brain THg (Fig. 3.2b) or OHg, but was negatively associated with IHg (p=0.03, r_s=-0.196). The significance of a curve fit at the 85th percentile in a quantile regression (p=0.05, b_0=525.42, b_1=-12.85) suggests that THg may be a limiting factor for GAD activity. Based on this analysis, concentrations of 10 µg/g and 20 µg/g brain THg would be associated with decreases in maximum

GAD activity of 25% and 50% compared to unexposed birds. For OHg, the 90th quantile was significant (p=0.03, b_0=646.64, b_1=-63.64) but quantile regression did not predict that IHg was a limiting factor for GAD activity. Binding to the NMDA receptor decreased in association with brain THg (p=0.005, r_p=-0.245) (Fig. 3.2c) and IHg (p=0.001, r_p=-0.282), but not OHg. Binding to the $GABA_A$ (benzodiazepine) receptor was not associated with brain THg (Fig. 3.2d), OHg, or IHg. Associations between brain Se and neurochemical receptors and enzymes were also investigated. GS activity and NMDA and GABA receptor binding did not correlate significantly with brain Se or the brain Hg:Se ratio. GAD activity was negatively correlated with brain Se (p=0.005, r_s=-0.430) but not the Hg:Se ratio. Neither age nor gender were significantly associated with any neurochemical parameter.

3.4 Discussion

The current study is only the second to investigate brain Hg levels in bald eagles, and we found that THg levels in these eagles from the US Great Lakes (0.2 µg/g to 34.01 µg/g) were comparable to those found in eagles from Canada (0.3 µg/g to 23 µg/g; Scheuhammer et al. 2008). Although no threshold has been derived for Hg's effects on the avian brain, levels found in the current study have been associated with neurochemical change in loons, eagles (Scheuhammer et al. 2008), and several wild mammals (Basu et al. 2007a, 2009a). Our results also support those of Scheuhammer et al. (2008) showing demethylation occurs in the bald eagle brain, particularly in birds with higher concentrations of THg. This is of interest because the form of the metal affects its toxicity, sequestration, and

elimination from the body (Clarkson et al. 2006). Also as in the Scheuhammer et al. (2008) study, Hg was correlated with Se and the Hg:Se ratio. Accumulation of Se represents an important detoxification mechanism (Clarkson et al. 2006), and that the Hg:Se ratio rarely exceeded 1 suggests that Se may offer some protection against Hg toxicity.

In our study, concentrations of THg in the liver averaged 7.97 µg/g and ranged from 0.47 µg/g to 61.61 µg/g. Bald eagles in British Columbia, Canada from 1987-1994 had a mean liver concentration of 11.8 µg/g and most birds fell in a range from 0.5 µg/g to 17.2 µg/g (Weech et al. 2003). Liver concentrations in adult bald eagles collected in Florida from 1987-1993 ranged from 2.17 µg/g to 42.07 µg/g (converted from dry weight; Wood et al. 1996). Eagles collected across Canada during the 1990s also had a similar range of 0.5 µg/g to 104 µg/g as well as similar relationships between THg and Se and the Hg:Se ratio (Scheuhammer et al. 2008). A conservative liver threshold for toxic effects in waterbirds is 16.7 µg/g (converted from wet weight assuming 70% moisture; Zillioux et al. 1993), which was exceeded by 14% of birds in our study. We measured on average 15 µg/g THg in breast and primary feathers. Mean feather concentrations in eagles in Idaho (Bechard et al. 2009) and three British Columbia reference sites (Weech et al. 2006) were 18.74 µg/g and 9.3 µg/g, 13 µg/g, and 14 µg/g, respectively. Concentrations in primary feathers collected across the Great Lakes from 1985-1989, which ranged from 3.6 µg/g to 48 µg/g (Bowerman et al. 1994), were slightly higher than those found in our more recent study. Though we lack information regarding habitat differences

that may explain disparities, this may suggest a decreasing trend in Hg contamination in eagles which is supported by findings that breast feather Hg levels declined 2.4% annually from 1991-2008 in eagles along Lake Superior (Dykstra et al. 2010). Scheuhammer (1991) suggested that levels above 20 μg/g in raptor feathers might be of concern. Evers et al. (2008) identified 40 μg/g in feathers as a threshold for adverse effects in loons. In our study 27% of eagles had levels over 20 μg/g, and 7% had levels over 40 μg/g in breast feathers. From proposed liver and feather thresholds, our regression equations relating these tissues to brain Hg predict a brain threshold for toxicity of approximately 4.5 μg/g, which is exceeded by 17% of eagles in this study. However, it must be noted that these thresholds do not consider dietary Se, which likely influences thresholds in individuals.

Although the brain is a key site for Hg toxicity, monitoring studies usually rely on levels in feathers and blood, which may be sampled non-lethally, to assess trends and health risks. Ideally, levels in these tissues would predict risks for neurotoxic effects. In this study we measured Hg in several tissues and evaluated liver, breast feathers, and primary feathers as predictors of levels in brain. As in previous eagle work (Scheuhammer et al. 2008), liver THg correlated highly with brain THg suggesting that levels in liver may give a fair indication of risk for neurotoxic effects. We found that breast feathers may be useful for predicting brain Hg concentrations, but caution should be used when extrapolating brain levels from breast feather levels as the correlation between brain and breast feather THg, though significant, was weaker than that with liver THg. Primary feather THg was not significantly correlated with brain THg and therefore may not be useful to

predict brain levels. These findings are similar to those of Hopkins et al. (2007) demonstrating that, although body feathers better predict tissue levels than do flight feathers, feathers are not always indicative of the Hg body burden in osprey (Hopkins et al. 2007). Nonetheless, feather collection is an important non-lethal technique for long-term monitoring of Hg exposure in live birds.

We also investigated spatial patterns in brain Hg concentrations and most notably found that birds in MI had higher levels than birds in other states. No other study has reported brain Hg levels in eagles from the Great Lakes, but a study of feathers sampled from 1985-1989 reported the lowest Hg levels in birds from Lake Erie and little variation in birds from the more western Great Lakes (Bowerman et al. 1994). Likewise, we found low brain Hg levels in birds from Ohio, which borders Lake Erie. However, we did find some brain Hg differences between birds in states bordering more western lakes. The discrepancy in spatial trends between the two studies may be due to temporal trends, as reported by Dykstra et al. (2010), habitat differences, or to the weak correlation between feather and brain Hg that we identified.

We examined relationships between Hg exposure and four neurochemical biomarkers, including three that were previously unstudied in wildlife. Consistent with a previous study on eagles (Scheuhammer et al. 2008), we observed a Hg-associated decrease in NMDA receptor levels. Hg toxicity is characterized by an increase in synaptic glutamate as reuptake by astrocytes is inhibited (Aschner 1996). Because extracellular glutamate is toxic (Albrecht et al. 1996), this decrease in NMDA receptors may be an adaptation to prevent excitotoxicity. When coupled

with several studies demonstrating a similar Hg-associated decrease in NMDA receptors (Basu et al. 2007b, 2009a; Scheuhammer et al. 2008), our results help support the notion that the NMDA receptor is a sensitive indicator of Hg's earliest neurological effects. We found no relationship between Hg and $GABA_A$ receptor benzodiazepine receptors, which are known to increase with Hg exposure in rats (Concas et al. 1983). We observed a slight, though significant increase in GS activity with brain THg levels. While this enzyme has been inhibited by Hg *in vitro* (Monnet-Tschudi et al. 1996), one rat study found a Hg associated increase *in vivo* (Kung et al. 1989). These disparate results may be explained by *in vitro* work showing that extracellular glutamate, which is common in Hg toxicity, increases GS expression (Lehmann et al. 2009). With GAD, we found a significant association with IHg, but not with OHg or THg. Results of previous *in vivo* studies vary, with some showing no effect of Hg treatment on GAD activity (Tsuzuki 1981; Concas et al. 1983) and others showing a decrease in activity (O'Kusky et al. 1985, 1987). However, these studies do not relate activity to brain levels of THg, OHg, or IHg. Both OHg and IHg inhibit GAD (Monnet-Tschudi et al. 1996; Basu et al. 2010) and IHg is more potent (Basu et al. 2010), which could explain our findings. Another interesting aspect to the relationship is that quantile regression suggests THg may be a limiting factor for GAD activity. This type of relationship with THg, which is represented by a wedge shaped plot, has been seen with hormone concentrations in minnows (Drevnick et al. 2003), productivity in loons (Burgess et al. 2008) and neurochemical enzyme activity in mink (Basu et al. 2007a), and indicates that Hg may impose an upper limit

on GAD activity. However, owing to the tremendous variability in enzyme activity further work is required to interpret the relationship between Hg and GAD.

In conclusion, this study demonstrates that many bald eagles in the Great Lakes region accumulate substantial levels of Hg and provides evidence that Hg is associated with neurochemical changes. Similar changes have been identified in mink (Basu et al. 2007b), otters (Basu et al. 2007a), polar bears (Basu et al. 2009a), loons, and eagles (Scheuhammer et al. 2008), but the relevance of these subclinical changes is unknown. Considering that Hg is known to affect learning, memory, and motor coordination (Evers et al. 2008; Bennett et al. 2007; Spalding et al. 2000; Kenow et al. 2010), which are in part regulated by glutamate and GABA (Scholes 1965, Gibbs et al. 2008), changes in these neurotransmitters may impact ecologically important behaviors in wildlife. Future work should focus on identifying a threshold for Hg's subclinical effects on the brain and clarifying the relationship between subclinical changes and relevant behavioral changes.

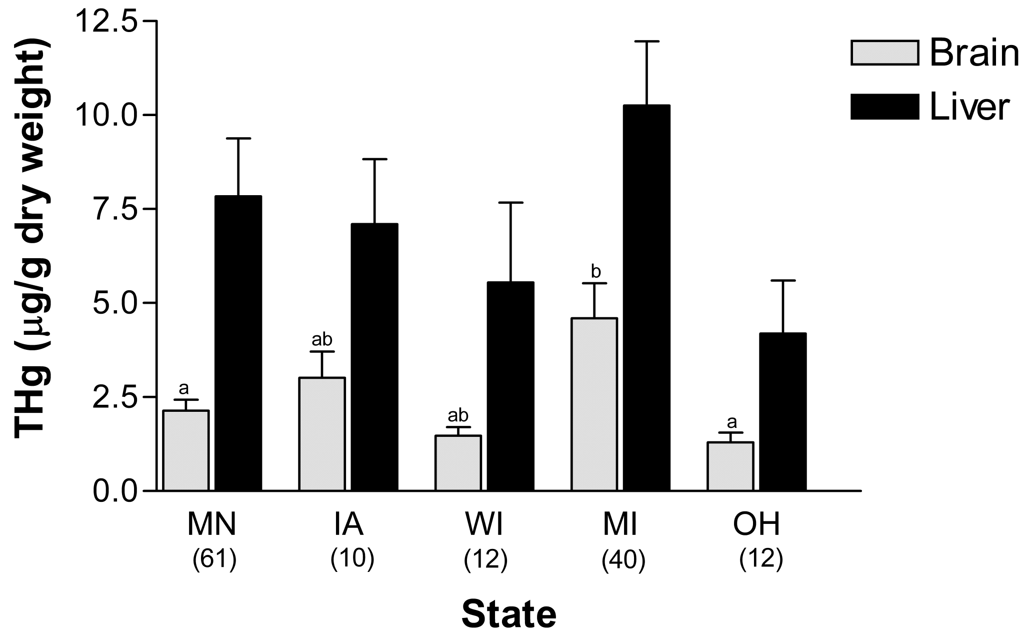

Figure 3.1 Total mercury (THg) in brain and liver of wild bald eagles from Minnesota (MN), Iowa (IA), Wisconsin (WI), Michigan (MI), and Ohio (OH) (2002-2010). Sample sizes are indicated in parenthesis. Bars represent mean ± SE and letters denote significant differences (bars that do not share a letter are significantly different) between states ($p<0.02$)

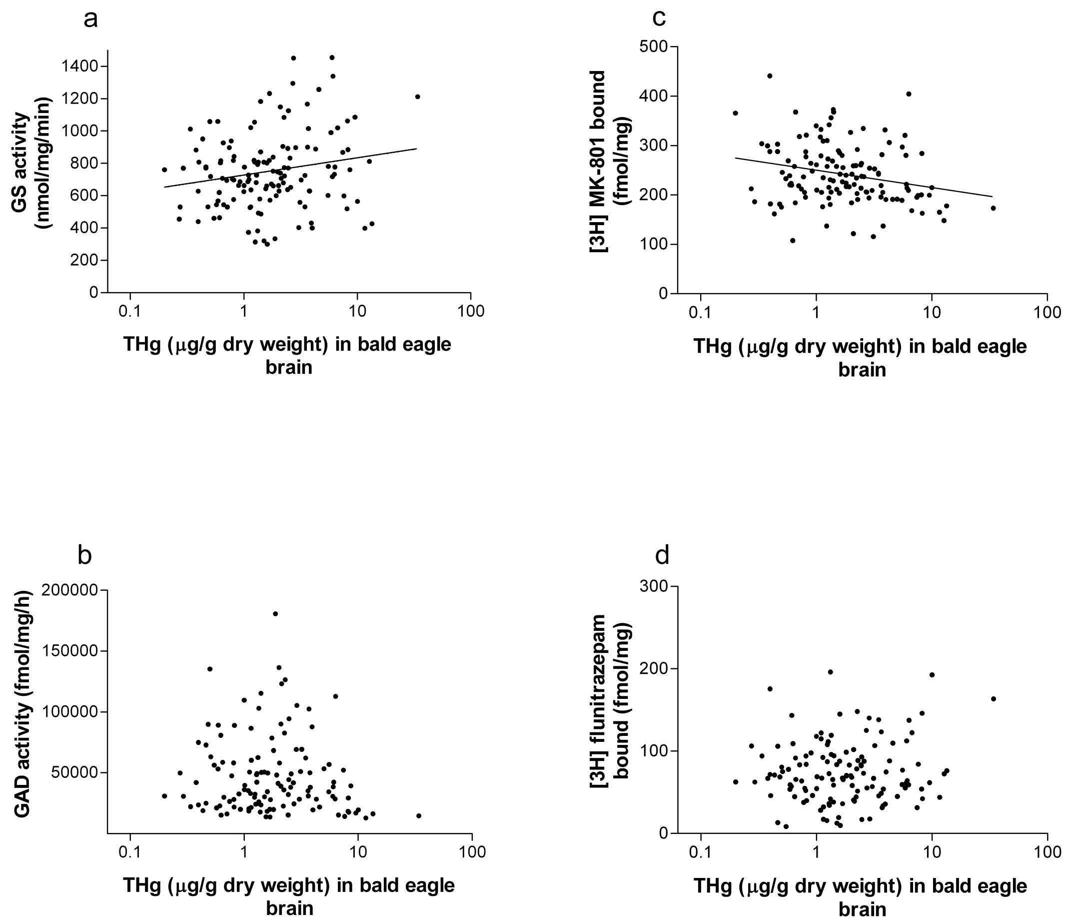

Figure 3.2 Mercury and neurochemistry in bald eagles. Associations between total mercury (THg) and (a) glutamine synthetase (GS) activity, (r_p=0.190, p=0.028, n=133), (b) glutamic acid decarboxylase (GAD) activity (r_s= -0.101, p=0.269, n=123), (c) N-methyl-D-aspartic acid (NMDA) receptor levels (r_p=-0.245, p=0.005, n=128), and (d) g-aminobutyric acid ($GABA_A$) benzodiazepine receptor levels (r_p=0.102, p=0.252, n=128) in the brain of wild bald eagles collected between 2002-2010). Dashed lines represent best fit linear regression

Table 3.1 Mercury in bald eagle brain and liver. Mean concentrations (± standard deviation) of total mercury (THg) (μg/g dry weight) and organic mercury (OHg, as a percentage of THg) in whole brain and liver from wild collected bald eagle carcasses collected between 2002-2010, stratified by sex and age class. Letters denote significant differences (p<0.01) between groups. Samples sizes are indicated in parenthesis

	Brain		Liver	
	THg (mg/g)	Percent OHg	THg (mg/g)	Percent OHg
All eagles	2.80 ± 3.78 (135)	64.09 ± 25.14 (134)	7.97 ± 10.51 (135)	59.33 ± 22.64 (125)
Sex				
Male	4.40 ± 6.78 (24)	58.84 ± 29.13 (24)	11.40 ± 13.90 (24)	49.18 ± 22.90 (23)
Female	2.71 ± 2.65 (45)	56.03 ± 25.17 (45)	8.81 ± 11.64 (45)	55.69 ± 19.56 (45)
Unkown	2.28 ± 2.67 (66)	71.6 ± 21.43 (65)	6.15 ± 7.72 (66)	66.31 ± 22.92 (57)
Age Class				
Nestling	0.52 ± 0.16 (8) [a]	80.94 ± 21.48 (8)	0.90 ± 0.33 (9) [a]	94.91 ± 11.25 (6)
Juvenile	1.03 ± 0.60 (11) [abc]	71.33 ± 20.85 (11)	2.42 ± 1.80 (11) [a]	74.46 ± 22.34 (10)
Subadult	2.67 ± 3.44 (20) [cd]	78.05 ± 11.96 (20)	8.78 ± 10.53 (20) [b]	63.74 ± 18.77 (20)
Adult	3.40 ± 4.52 (75) [de]	56.72 ± 27.35 (75)	9.46 ± 12.03 (75) [b]	53.64 ± 19.82 (73)
Unknown	2.59 ±1.89 (21)	67.01 ± 19.76 (20)	7.97 ± 10.51 (20)	56.99 ± 26.72 (16)

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Chapter 4

Distribution of mercury in the white leghorn chicken embryo and hatchling following air cell egg injection

4.1 Introduction

Methylmercury (MeHg) is a well established environmental toxicant known to affect brain (Heinz et al. 1976, Pass et al. 1975, Spalding et al. 2000) and kidney (Pass et al. 1975, Spalding et al. 2000) histopathology, neurochemistry (Rutkiewicz et al. 2011, Scheuhammer et al. 2008), and behavior and motor skills (Evers et al. 2008, Kenow et al. 2010, Spalding et al. 2000) in birds. Reproduction is a particularly sensitive endpoint, and reduced productivity has been associated with MeHg exposure in several species in the field (Evers et al. 2008) and laboratory (Albers et al. 2007). Though decreased hatching success may be partially attributed to alterations in parental care, direct effects on the embryo may also contribute. Wild adult birds routinely accumulate MeHg through their diet (Evers et al. 2008, Rutkiewicz et al. 2011), and during egg production in females this Hg becomes incorporated into the albumen fraction (Heinz et al. 2004) to produce eggs containing high levels of Hg (Evers et al. 2003, Heinz et al. 2004). Therefore, embryos receive continuous exposure to Hg from the time of fertilization through sensitive developmental stages and hatching.

Egg injection methods have been used to study the developmental toxicity of a variety of environmental toxicants, including TCDD (Cohen-Barnhouse et al. 2011), PCBs (Grasman et al. 2001), and PBDEs (McKernan et al. 2009) in birds, and these methods have been well-optimized to study MeHg's toxicity to the developing avian embryo (Heinz et al. 2006). This technique involves injecting fertilized eggs with MeHg, so that the developing embryo is exposed under a controlled setting in the absence of other contaminants of environmental factors. Such studies have been used to compare relative embryonic toxicity (Heinz et al. 2009) and teratogenesis (Heinz et al. 2011) of MeHg between several avian species, to study the effect of MeHg on mallard hatching success (Heinz et al. 2012), and to study the effect of MeHg on common loon survival, organ masses, and behavior (Kenow et al. 2011). Similarly, these air cell injection techniques may be used to study physiological effects of Hg in avian embryos.

Despite the abundance of information regarding MeHg toxicity in birds, including several egg injection studies, very little is known about how MeHg distributes in the avian embryo. The time course of MeHg accumulation and distribution across tissues determine potential windows and sites of toxicity in the embryo. The studies described here aim to use air cell injections to gain a better understanding of the toxicokinetics of Hg in the avian embryo. Most of this work involves the white leghorn chicken (*Gallus gallus domesticus*), which is potentially a useful model for avian toxicity studies because it is moderately sensitive to MeHg (Heinz et al. 2009), its normal physiology is fairly well understood, and eggs may be easily obtained commercially. The primary goal of this work is to determine the

distribution of Hg in embryonic and hatchling tissues during different stages of development following *in ovo* exposure to a range of MeHg doses via air cell injection. Secondary goals include comparing the distribution of Hg in the chicken embryo following exposure to different forms of Hg, and comparing tissue distribution in the chicken embryo to that in the Japanese quail (*Coturnix japonica*) embryo. This work provides insight into potential sites of toxicity and Hg sequestration within the avian embryo and identifies possible developmental periods most susceptible to Hg's toxic effects, therefore laying the foundation for the use of this model as potential tool for studying Hg associated physiological changes in birds.

4.2 Methods

4.2.1 Animals

The data presented here were compiled from a series of egg injection studies involving fertilized white leghorn chicken eggs or Japanese quail eggs (Michigan State University Poultry Teaching and Research Center). Eggs were injected with Hg in the form of methylmercury chloride (MeHgCl) (Sigma-Aldrich, 13% Cl) in corn oil, methylmercury cysteine (MeHgCys) in corn oil (prepared with twofold molar excess of cysteine), or mercury chloride ($HgCl_2$) (Sigma-Aldrich, ≥ 99.5%) in corn oil (initially dissolved in DMSO to yield 1% DMSO in final solution). Specific details regarding doses, sampling timepoints, and tissue sampling are presented in Table 4.1.

Air cell injection methods were adapted from Heinz et al (2009) but in order to better mimic maternal deposition, eggs were injected prior to incubation (day 0). For all studies, eggs were weighed and candled on day 0 to locate the air cell, which was then wiped with 70% ethanol. Two holes were drilled with a Dremel tool, and eggs were injected with 0.5 µl/g egg of MeHgCl, MeHgCys, or $HgCl_2$ (Sigma Aldrich) dissolved in corn oil. A vehicle control, injected with corn oil, was included in all studies. A non-inject control was included in studies 1 and 5. Holes were sealed with hot glue, and after remaining upright for 30 minutes the eggs were placed horizontally in an egg incubator (GQF Sportsman 1502) at 37.7°C with 55-60% humidity. At sampling timepoints, embryos were removed and euthanized by decapitation, and tissues were dissected and frozen immediately on dry ice. Tissues were stored at -80°C (brain) or -20°C (all other tissues) until Hg analysis. All aspects of this study were approved by the University of Michigan Committee on Use and Care of Animals.

4.2.2 Hg Analysis

Total Hg was measured in a small subsection (15-50mg) of wet brain, liver, heart, muscle, kidney, and yolk and fresh weight feathers. Feathers were washed three times in acetone then deionized water and dried at room temperature for 24 hours prior to analysis. For organic mercury (OHg) analysis in brains, micro-scale extractions were performed on 10-20 mg brain tissue according to previously published methods (Nam et al. 2011). Total Hg and OHg were measured by thermal decomposition, amalgamation, and atomic absorption spectrophotometry in a

Direct Mercury Analyzer (DMA-80, Milestone Inc, CT) as we have previously detailed (Rutkiewicz et al. 2011). Residue information is presented on a wet weight basis. Concentrations are reported as mean ± standard deviation. Standard Reference Materials (SRMs) (TORT-2, DOLT-4, National Research Council of Canada) were included to monitor accuracy and precision. Average accuracy was within 3% of the certified value and average precision (% relative standard deviation) was 9%. Detection limits varied by day and tissue type, but other than some control samples, no sample fell below its respective detection limit.

4.2.3 Statistics

Statistical analyses were performed using IBM SPSS Statistics (V.19.0, New York). A p-value of less than 0.05 was considered to be significant. Descriptive statistics (mean, standard deviation, and range) were calculated for Hg concentrations in tissues. Kruskal-Wallis (for comparisons between multiple groups), and Mann-Whitney (for comparisons between two groups) tests were used to assess significant differences in tissue THg concentrations between treatment groups, species, and Hg forms. Spearman correlation was used to assess the relationship between THg and OHg in the brain.

4.3 Results

4.3.1 Tissue Distribution in Embryos

Mercury was detected in all tissue types from embryos and hatchlings from eggs injected with MeHgCl. Total Hg concentrations in tissues from both vehicle and

non-inject controls were low, and did not differ significantly from one another in any tissue (Table 4.2). Total Hg concentrations in whole embryos from eggs injected with 6.4 μg/g egg were low at embryonic days 4 (0.05 ± 0.03 μg/g, n=4) and 7 (0.05 ± 0.02 μg/g, n=3). Total Hg concentrations in all tissues increased steadily throughout embryonic development, with the most rapid increase in feathers between days 14 and 16 and in soft tissues after day 16 (Fig. 4.1). Total Hg concentrations in all tissues from day 19 embryos from eggs injected with 0.17, 0.62, 2.0, 6.4 μg/g egg increased in a dose dependent manner with treatment group. Tissue THg concentrations in day 19 embryos are presented in Table 4.2. Relative rankings of tissue concentrations were similar at all stages of embryonic development and generally did not differ based on treatment group. Total Hg concentrations were highest in feathers, followed by liver, kidney, muscle, heart, and brain. Total Hg concentrations did not differ significantly between liver, kidney, brain, heart, and muscle, but were significantly higher in feathers than in all other tissues.

4.3.2 Tissue Distribution in Hatchlings

In addition to assessing THg levels throughout embryonic development, we also characterized levels in day 1 and day 7 hatchlings. Total Hg concentrations in soft tissues continued to increase sharply through hatching, and plateaued between post-hatch days 1 and 7 (Fig. 4.1). Total Hg concentrations in feathers continued to increase through post-hatch day 7. Relative rankings of tissue THg concentrations in hatchlings were similar to that in embryos, but while levels in liver slightly

exceeded those in kidney during embryonic development, levels in kidney slightly exceeded those in liver at days 1 and 7 post-hatch. Mean brain (cerebrum) THg concentrations increased from 1.9 ± 0.24 μg/g (n=3) in day 19 chicken embryos to 4.0 ± 0.88 μg/g (n=4) in day 1 hatchlings, and 4.3 ± 0.67 μg/g (n=7) in day 7 hatchlings. In all treatment groups, THg concentrations did not differ significantly between cerebrum, cerebellum, and optic lobe (Table 4.3) in embryos or hatchlings. On average, 89% (range: 43%-110%) of the Hg in the brain was OHg, and there was no relationship between THg and the percent OHg in the brain. Chicks had substantial quantities of unabsorbed yolk at embryonic day 19 and post-hatch day 1. Concentrations of THg in unabsorbed yolk were 13.02 ± 4.72 μg/g (n=3, mean yolk remaining=13.66g) at embryonic day 19, and 26.91 ± 12.99 μg/g (n=3, mean yolk remaining=5.20g) at post-hatch day 1.

4.3.3 Tissue Distribution in Day 15 Quail Embryos

To explore for inter-species differences, we ran a pilot study on Japanese quail. Relative tissue distribution of THg in day 15 quail embryos was similar to that seen in day 19 chicken embryos, with the lowest concentrations in brain, muscle, and heart, higher concentrations in liver and kidney, and the highest concentrations in feathers and unabsorbed yolk (Fig. 4.2). Unlike in the chicken embryo, concentrations in kidney were slightly higher than those in liver. Absolute concentrations of THg in day 15 quail embryo tissues were also similar to those found in day 19 chicken embryos exposed *in ovo* to an identical dose. However,

concentrations in quail heart approached those in kidney and liver. Concentrations did not differ significantly between quail and chicken for any tissue.

4.3.4 Methylmercury Cysteine and Mercury (2) Chloride Injection

Here we explored two additional forms of Hg in a second pilot study. Distribution of THg in the day 19 chicken embryos from eggs injected with MeHgCys was similar to that seen in embryos from eggs injected with the same amount of MeHgCl (Fig. 4.3). The lowest concentrations were measured in the brain heart, and muscle, while levels were higher in the kidney and liver. High levels were detected in the yolk and feathers. Tissue THg concentrations did not differ significantly from those seen with MeHgCl injection for any tissue.

Total Hg concentrations were fairly low in all tissues of day 19 chicken embryos following injection of $HgCl_2$ into the air cell (Fig. 4.3). Some accumulation was observed in the kidney, liver, and feathers, but not to the extent that was seen following MgHgCl or MeHgCys injection. Differences from THg concentrations seen with MeHgCl injection were marginally significant ($p=0.06$) for brain, liver, heart, feather, muscle. Only yolk contained concentrations of THg similar to those found after injection of methylated forms of Hg.

4.4 Discussion

Mercury exposure is associated with decreased reproductive success in birds, but most studies to date have focused on impacts to parents (Evers et al. 2008) rather than developing embryos and hatchlings to explain these observations.

Furthermore, those that do focus on developmental effects to the offspring rarely look beyond mortality and gross deformities (Heinz et al. 2009, 2011) to identify physiological changes underlying toxicity. The primary goal of our current work was to evaluate levels of Hg in various tissues to gain a better understanding of potential sites of toxicity and sequestration of Hg in embryos and hatchlings exposed developmentally to MeHg. Our findings demonstrate that distribution of Hg in the chicken embryo following *in ovo* MeHg exposure via air cell injection is similar to that seen in chicks from mothers experimentally fed MeHg dicyandiamide (Tejning et al. 1967), as well as in older birds exposed to MeHg through the diet (Bennett et al. 2009, DesGranges et al. 1998, Henny et al. 2002).

Sequestration in certain tissues can sometimes protect from Hg toxicity by preventing the metal from reaching target organs. As in older birds, this may occur in embryos and hatchlings as they accumulate Hg in liver, kidney, and feathers, which are organs believed to be important in Hg sequestration. High levels of Hg in the liver and kidney, relative to other soft tissues, have been documented in wild black-crowned night heron, snowy egret, and double-crested cormorants nestlings and adults (Henny et al. 2002), osprey nestlings (DesGranges et al. 1998), and common tern chicks (Becker et al. 1993), and also in adult American kestrels exposed to MeHgCl experimentally (Bennett et al. 2009). Eagles-Smith et al. (2009) have found that waterbird chicks demethylate MeHg in the liver regardless of the concentration. This coupled with the high concentrations that we measured in liver may suggest that embryos and hatchlings demethylate MeHg and sequester it in the liver in a complex with selenium, which is thought to be a protective strategy that

has been employed by wild birds (Henny et al. 2002, Scheuhammer et al. 2008). Mercury induces the metal-binding protein metallothionein (MT) (Nam et al. 2008) and may bind to it in the kidney of birds (Prasada et al. 1989). Our findings of high Hg concentrations in kidney may suggest that this occurs in embryos and hatchlings, which may protect from Hg's neurotoxic effects. The high concentrations of Hg in feathers, relative to soft tissues, that we observed is similar to that seen in older birds (DesGranges et al. 1998, Rutkiewicz et al. 2011), and demonstrates that incorporation of MeHg into growing feathers could be an important sequestration mechanism in embryos and chicks.

Embryos and hatchlings may also share with adult birds common sites of toxicity. High levels of Hg in the kidney, which in hatchlings from eggs injected with 6.4 μg/g egg exceeded the minimum kidney concentrations associated with kidney lesions in mallard ducks fed MeHgCl (Pass et al. 1975), indicate that birds at these early life stages may be susceptible to the renal toxicity seen in older birds. We also saw that Hg accumulates in brain as has been seen in adults of other species exposed through the diet in the field (DesGranges et al. 1998, Rutkiewicz et al. 2011) and laboratory (Bennett et al. 2009, Heinz et al. 1976), which may leave embryos and hatchlings exposed developmentally at risk for neurological effects such as the pathological changes seen in birds exposed in the laboratory (Pass et al. 1975) or neurochemical changes seen in birds in the field (Rutkiewicz et al. 2011, Scheuhammer et al, 2008). In our study involving MeHgCl, after injection with 6.4 μg/g egg, concentrations of Hg in the brain at embryonic day 19 were approximately 32% and at post-hatch days 1 and 7 were 70-75% of the concentrations associated

with brain lesions in mallard ducklings exposed developmentally via natural maternal deposition (Heinz et al. 1976). Mean concentrations in the brain of day 7 hatchlings were within the range for which brain lesions were detected (Pass et al. 1975). Based on our studies here, the three brain regions studied accumulate similar levels of Hg and are all potentially at risk for neurotoxic effects.

Increased knowledge of the time course of Hg accumulation in embryonic tissues may provide insight into potential windows of susceptibility to its toxic effects. We found that Hg concentrations in all tissues were fairly low until approximately incubation day 16, and then increased rapidly through one day after hatch. This may be explained by yolk absorption later in development. Because yolk absorption increases rapidly shortly before hatch (Romanoff 1960) and the yolk had accumulated high concentrations of Hg by this late stage of development, the embryo received a sudden surge in Hg exposure during the latest embryonic stages. This may indicate that the embryo is particularly at risk for Hg toxicity during the critical pipping and hatching stage, as well as during the first week post-hatch as up to 40% of the yolk is absorbed during this time (Romanoff et al. 1960). In fact, Tejning et al. (1967) report that chicks exposed to *in ovo* after mothers were fed MeHg dicyandiamide often die shortly after hatch, perhaps due to absorption of yolk although it should be noted that we did not observe this phenomenon in out studies. During this period of yolk absorption, incorporation of Hg into feathers, which begin their most rapid growth during the latest stages of embryonic development (Meyer et al. 2008), offers some protection from toxicity and likely helps to explain why levels of Hg in soft tissues appear to stabilize between post-hatch days 1 and 7.

Similar depuration has been previously been demonstrated in common tern and herring gull chicks from contaminated environments (Becker et al. 1994) as well as in common loon chicks experimentally exposed to Hg (Fournier et al. 2002), and may help minimize potential toxicity at this timepoint.

One of our objectives with this work was to further evaluate air cell injection methods and the chicken model as tools for studying Hg toxicity in birds. Heinz et al. (2006) have optimized injection methods and established that this technique results in Hg accumulation in the albumen, where the majority of Hg would be found following natural maternal deposition (Tejning et al. 1967). They also found that these methods are an excellent way to compare mortality rates between species (Heinz et al. 2009). In the current study, we discovered that air cell injection of MeHgCl and MeHgCys produce fairly high and similar levels of Hg in tissues like kidney and brain, which are known targets of Hg toxicity. Considering that we observed tissue Hg distributions similar to those seen with natural exposure to Hg, including in target organs, egg injections may be a convenient means for assessing Hg risks in birds. As in feeding studies, sensitive tissues receive high exposures, but egg injection studies offer a more controlled setting for exposure that minimizes the impact of environmental factors therefore allowing investigators to attribute observed toxicity to Hg exposure. Our findings here support the use of MeHgCl or MeHgCys, but not $HgCl_2$, which is not a form relevant in nature, in egg injections for the study of Hg toxicity in embryos and hatchlings and for mechanistic studies of Hg's toxicity in known target organs. However, when interpreting results from such

studies it is important to consider that effects of developmental exposure in embryos and hatchlings may differ from those in adults with long term exposures.

In summary, we found that following egg injection with MeHgCl, tissue accumulation of Hg in chicken embryos and hatchlings is similar to that see in adult birds exposed to Hg through the diet. Liver and kidney accumulated high concentrations of Hg, making them sites of sequestration and perhaps toxicity. Though lower than concentrations in liver and kidney, concentrations in brain did reach levels considered to be neurotoxic, and high concentrations in feathers suggest feather growth is an important means of depuration during development. Rapid increases in tissue concentrations during later stages of embryonic development indicate that embryos may be most susceptible to toxicity during pipping and hatch. Our studies involved injection with levels of MeHg that were both similar to and higher than those found in eggs of most species in the field (reviewed in Wolfe et al. 1998). Our findings from these studies provide insight into potential sites and time periods for Hg toxicity, and help to evaluate egg injection methods as a tool for mechanistic studies of Hg toxicity studies in birds.

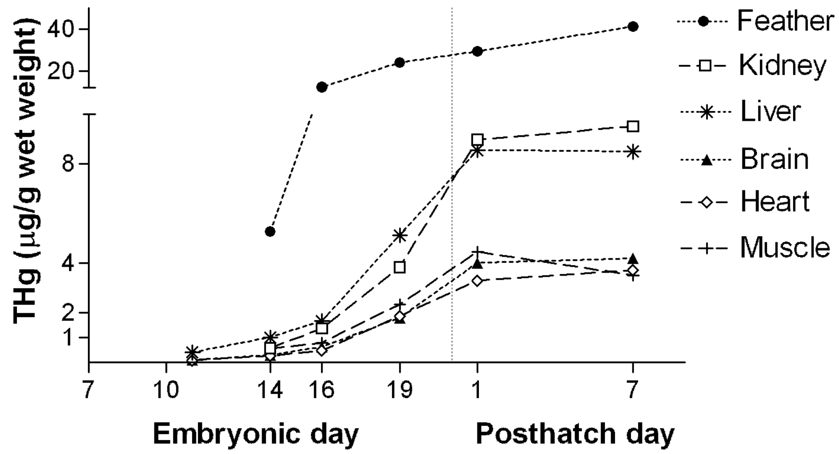

Figure 4.1 Total mercury (THg) concentrations (µg/g wet weight) in tissues of chicken embryos and hatchlings from eggs injected with methylmercury chloride (6.4 µg/g egg) in corn oil on incubation day 0. Sample sizes range from 3-7. Vertical line represents day of hatch. Data presented were derived from Study 2 (Table 4.1)

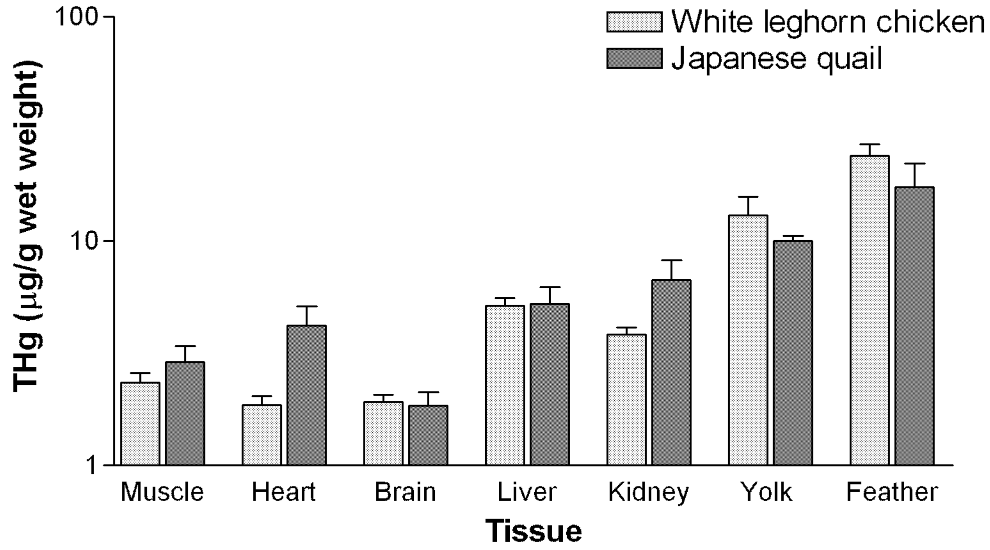

Figure 4.2 Mercury distribution in chicken and quail embryos. Total mercury (THg) concentrations in tissues from day 19 white leghorn chicken embryos (n=3) and day 15 Japanese quail embryos (n=7) from eggs injected with 6.4 µg/g egg methylmercury chloride in corn oil at incubation day 0. Bars represent mean ± SD. Total Hg concentrations did not differ significantly between species in any tissue

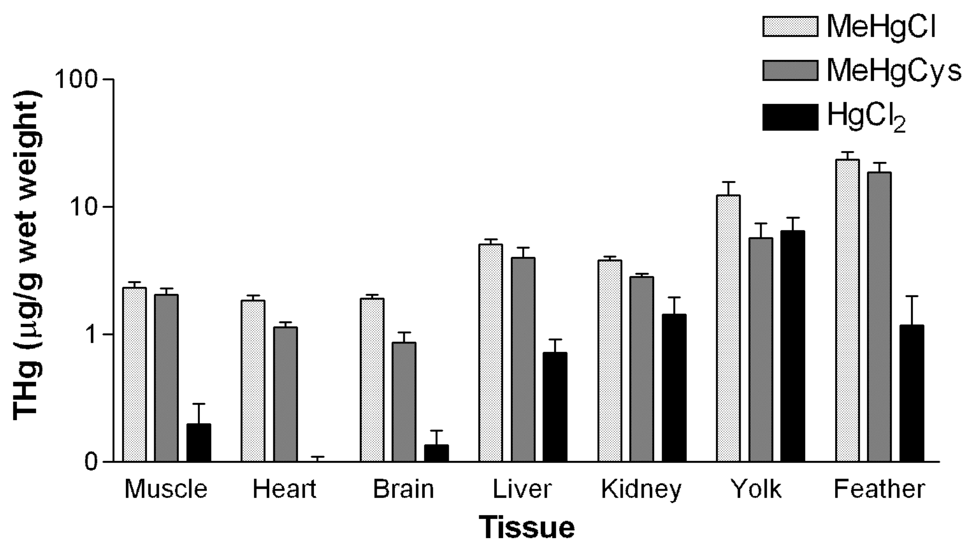

Figure 4.3 Comparison of tissue mercury in chicken embryos after injection of MeHgCl, MeHgCys, or $HgCl_2$. Total mercury (THg) concentrations in tissues from day 19 white leghorn chicken embryos from eggs injected with 6.4 µg/g egg methylmercury chloride (MeHgCl) (n=3), methylmercury cysteine (MeHgCys) (n=4), or mercury (2) chloride ($HgCl_2$) (n=4) in corn oil at incubation day 0. Bars represent mean ± SD

Table 4.1 Experimental conditions and sampling details for mercury egg (air cell) injection studies

	Study 1	Study 2	Study 3	Study 4	Study 5
Species	chicken	chicken	chicken	chicken	quail
Toxicant	MeHgCl	MeHgCl	MeHgCys	$HgCl_2$	MeHgCl
Doses (μg/g egg)	0, 0.17, 0.62, 2.0, non-inject	0, 6.4	0, 3.2, 6.4	0, 3.2, 6.4	0, 3.2, 6.4, non-inject
Sampling timepoints (embryonic or post-hatch day)	19	4, 7, 10, 14, 16, 19, post-hatch 1, post-hatch 7	19	19	15
Tissues sampled	cerebrum, cerebellum, optic lobe, heart, muscle, liver, feather, kidney	whole embryo (d4,7), whole brain (<d19), cerebrum (≥d19) , cerebellum (≥d19), optic lobe(≥d19), heart, muscle, liver, feather, kidney, yolk	brain (whole), heart, muscle, liver, feather, kidney, yolk	brain (whole), heart, muscle, liver, feather, kidney, yolk	brain (whole), heart, muscle, liver, feather, kidney, yolk

Table 4.2 Mercury concentrations in chicken embryos after methylmercury chloride egg injection. Total mercury concentrations [mean±SD/(range,*n*)] (µg/g wet weight) in tissues from day 19 chicken embryos from uninjected eggs or eggs injected with corn oil (control) or a range of doses of methylmercury chloride in corn oil on incubation day 0

	Non-inject[1]	**Control[1]**	**0.17 µg/g[1]**	**0.62 µg/g[1]**	**2.03 µg/g[1]**	**6.4 µg/g[2]**
CEREBRUM (µg/g)	0.003±0.002 (0.0008-0.008, *12*)	0.003±0.002 (0.0009-0.008, *13*)	0.053±0.024 (0.002-0.076, *13*)	0.194±0.088 (0.001-0.295, *13*)	0.740±0.271 (0.275-1.223, *14*)	1.918±0.235 (1.734-2.184, *3*)
LIVER (µg/g)	0.002±0.001 (0.0006-0.005, *12*)	0.003±0.001 (0.0008-0.004, *14*)	0.171±0.112 (0.004-0.304, *14*)	0.692±0.211 (0.151-0.923, *13*)	2.452±1.010 (0.003-3.395, *14*)	5.136±0.755 (4.131-5.794, *3*)
KIDNEY (µg/g)	0.003±0.002 (0.0006-0.005, *12*)	0.006±0.014 (0.001-0.048, *12*)	0.120±0.049 (0.008-0.181 *14*)	0.406±0.179 (0.010-0.574, *13*)	1.718±0.499 (0.864-2.713, *14*)	3.836±0.493 (3.313-4.292, *3*)
HEART (µg/g)	0.001±0.0004 (0.001-0.002, *10*)	0.002±0.002 (0.0006-0.008, *13*)	0.090±0.106 (0.004-0.424 *14*)	0.232±0.127 (0.014-0.419, *13*)	0.814±0.379 (0.319-1.757, *14*)	1.861±0.302 (1.580-2.181, *3*)
MUSCLE (µg/g)	0.003±0.004 (0.0007-0.014, *12*)	0.002±0.001 (0.0008-0.005, *13*)	0.094±0.038 (0.007-0.155 *14*)	0.282±0.154 (0.008-0.473, *12*)	1.245±0.484 (0.463-2.185, *14*)	2.347±0.418 (1.896-2.721, *3*)
FEATHER (µg/g)	0.025±0.019 (0.005-0.059, *14*)	0.020±0.012 (0.006-0.043, *14*)	0.623±0.360 (0.009-1.188 *14*)	2.759±1.075 (1.462-5.826, *13*)	9.895±3.723 (5.169-20.923, *14*)	24.050±5.118 (18.342-28.230, *3*)

[1] Number indicates that values were obtained from Study 1 (Table 4.1)
[2] Number indicates that values were obtained from Study 2 (Table 4.1

Table 4.3 Mercury distribution in chicken brain. Total mercury concentrations (mean±SD) in brain regions of chicken embryos and hatchlings from eggs injected with methylmercury chloride in corn oil on incubation day 0. Sample sizes are indicated in parenthesis. Italicized values are for embryos from eggs injected with corn oil vehicle and non-italicized values are for embryos injected with 6.4 μg/g egg methylmercury chloride. Total Hg was significantly lower in vehicle control chicks compared to dosed chicks, and did not vary significantly across brain regions at any sampling point

	Day 19 (embryonic)	**Day 1 (post-hatch)**	**Day 7 (post-hatch)**
Cerebrum	0.02±0.01 (2) 1.92±0.24 (3)	0.01±0.01 (5) 3.98±0.88 (4)	0.04±0.02 (6) 4.33±0.67 (7)
Cerebellum	0.04±0.01 (2) 1.80±0.24 (3)	0.01±0.01 (5) 3.99±0.77 (4)	0.03±0.01 (6) 3.57±0.62 (7)
Optic lobe	0.04±0.00 (2) 1.69±0.25 (3)	0.01±0.01 (5) 3.91±0.56 (4)	0.02±0.00 (6) 4.73±0.79 (7)

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Chapter 5

Pathology, neurochemistry and behavior in the white leghorn chicken embryo and hatchling following air cell injection of methylmercury chloride

5.1 Introduction

Methylmercury (MeHg) is a toxic metal with neurological impacts in birds ranging from subtle biochemical changes to structural and behavioral changes. Exposure has been linked to reproductive impairment in captive (Albers et al. 2007), and wild birds (Burgess et al. 2008, Heath et al. 2005, Hill et al. 2008), and these changes may stem from MeHg associated changes in cognitive and motor skills. For example, in the laboratory, dietary MeHg has been associated with altered hunting, and shade seeking behavior (Bouton et al. 1999), and righting response and coordination (Spalding et al. 2000) in great egret chicks, altered righting and fright responses in common loon chicks (Kenow et al. 2010) and impaired balance in adult American kestrels (Bennett et al. 2007). Brain lesions have also been found in the cerebellum of adult mallards (Pass et al. 1975), the cerebellum and medulla oblongata of juvenile northern goshawks (Borg et al. 1970), the cerebellum of adult American kestrels (Bennett et al. 2007), and the midbrain, cerebellum, and cerebrum of great egret chicks (Spalding et al. 2000) fed MeHg contaminated diets.

Before inducing clinical toxicity, lower levels of MeHg exposure may first induce changes in neurochemical biomarkers, which include receptors and enzymes

involved in neurotransmission (Manzo et al. 1996). Though neurochemical receptors were not associated with brain Hg in adult common loons and herring gulls with low exposures (Rutkiewicz et al. 2010, Hamilton et al. 2011), several receptors and enzymes were associated with brain Hg in adult common loons and bald eagles with higher exposures (Rutkiewicz et al. 2011, Scheuhammer et al. 2008). While laboratory studies have found that lesions occur with brain Hg concentrations as low as 0.9 μg/g in adult mallards (Pass et al. 1975), much less is known about thresholds associated with subclinical neurochemical changes in birds. Furthermore, no studies have attempted to clarify the relationships between Hg exposure, neurochemical change, and behavioral change. An improved understanding of these relationships could help to establish neurochemical biomarkers as important tools for avian risk assessment.

Changes in receptors and enzymes involved in glutamate and γ-aminobutyric acid (GABA) neurotransmission may be particularly attractive biomarkers because these are the primary excitatory and inhibitory neurotransmitters in vertebrates (Ozawa et al. 1998, Reis et al. 2009). Disruption of the many components of either system can disturb the balance between excitation and inhibition (Reis et al. 2009), thereby resulting in excitotoxicity that may lead to neuronal death (Fitsanakis et al. 2005). As in other vertebrates (Ozawa et al. 1998, Reis et al. 2009), these neurotransmitters are involved in brain development (Andaloro 1998, Sato et al. 2005), learning (Gibbs et al. 2005, 2008), and motor coordination (Scholes 1965) in birds. Given that these are ecologically relevant behaviors and changes may impact an individual's ability to survive and reproduce, using receptors and enzymes

involved in glutamate and GABA as biomarkers may help to warn of important behavioral changes. Changes in several components of these pathways have been found in vitro and in mammalian studies (Basu et al 2007a, 2010, Concas et al 1983, Kung et al. 1989, Monnet-Tschudi et al. 1996), and associations between brain Hg and the *N*-methyl-D-aspartate (NMDA) receptor and the enzymes glutamine synthetase (GS) and glutamic acid decarboxylase (GAD), have been identified in wild adult common loons and bald eagles (Rutkiewicz et al. 2011, Scheuhammer et al. 2008).

Despite the fact that the embryonic brain is considered to be particularly sensitive to environmental neurotoxicants, including MeHg (Grandjean et al. 2006), and that embryonic development is a critical period for the formation of neurochemical systems, the brain structure, and behaviors (Mochida et al. 2009, Pearson 1972, Rogers 1993), relatively few studies have explored the effects of developmental MeHg exposure on the avian brain. Heinz et al. (1976) have shown that *in ovo* MeHg exposure induces brain lesions in mallard optic lobe and cerebellum (Heinz et al. 1976), and Kenow et al. (2011) have shown that *in ovo MeHg* exposure is associated with impaired righting response in loons, but to date no studies have investigated the effects of *in ovo* MeHg exposure on neurochemistry in birds. Egg injections may be useful for studying developmental neurotoxicity of MeHg under controlled settings. Following injection into fertilized eggs, embryos can be exposed to MeHg throughout development, and effects may be attributed to that exposure without having to consider confounding factors like other contaminants and environmental stressors. This technique has been used to study

the embryotoxicity (Heinz et al. 2009), teratogenicity (Heinz et al. 2011) and effects of MeHg on hatching (Heinz et al. 2012, Kenow et al. 2011) in several species, as well as on behavior in loon chicks (Kenow et al. 2011). Embryos and hatchlings exposed to MeHg via this technique accumulate Hg in their tissues, including the brain, in a manner similar to that seen in naturally exposed birds (Chapter 4), suggesting that egg injections may be a good way to study MeHg associated changes in an embryonic model.

The objective of this work is to utilize air cell egg injections to increase our understanding of the neurological impacts of developmental exposure to MeHg in birds. We looked at neurological changes at multiple tiers, assessing both the subclinical (i.e., neurochemistry) as well as the clinical impacts (i.e., neuropathology and neurobehavior) in a series of experiments primarily involving methylmercury chloride (MeHgCl) injection into white leghorn chicken (*Gallus gallus domesticus*) eggs. We investigated pathological changes in day 19 embryos, and measured levels of the NMDA and $GABA_A$ (benzodiazepine) receptors and the GS and GAD enzymes, which have previously been studied in wild bald eagles (Rutkiewicz et al. 2011), in both whole brain and discrete brain regions (cerebrum, cerebellum, and optic lobe) during several stages of embryonic and post-hatch development to get a full understanding of MeHg's potential impacts on neurochemistry. An experiment on Japanese quail (*Coturnix japonica*) embryos was included to compare neurochemical responses between species, and one study involving injection of methylmercury cysteine (MeHgCys) rather than MeHgCl was included to determine if responses differed based on the form of the MeHg. In addition, we evaluated the impacts of *in*

ovo MeHg exposure on hatchling righting response, balance on level and angled beams, and startle response in order to improve our understanding of effects on skills that may be important for survival in ecological settings. This work will offer information about the potential effects of developmen tal Hg exposure on brain neurochemistry and behavior in birds.

5.2 Methods

5.2.1 Animals

The neurochemical and behavioral data presented here were compiled from a series of six egg injection studies designed to investigate various aspects of the relationship between MeHg exposure and neurological change. Egg injection methods were adapted from Heinz et al. (2009) and are described in detail in Chapter 4. For all studies, eggs were injected with MeHgCl or MeHgCys in corn oil at day 0. A vehicle control was included in all studies and a non-inject control was included in studies 1 and 6. For studies on embryos, eggs were incubated for 10-19 days and embryos were euthanized by decapitation, and brains (either whole or dissected into cerebrum (CER), cerebellum (CBL), and optic lobe (OL)) were frozen immediately on dry ice for neurochemical assays. For studies on hatchlings, chicks were allowed to hatch and were housed in a brooder (90-100˚C). At post-hatch days 1 and 7 behavioral tests were performed, chicks were euthanized by decapitation, and brains (CER, CBL, and OL) were frozen on dry ice. Tissues were stored at -80°C (brain) until Hg and neurochemical analysis. Specific details regarding doses, sampling timepoints, and tissue sampling are presented in Table 5.1.

5.2.2 Mercury Analysis

Total Hg (THg) was measured in a Direct Mercury Analyzer (DMA-80, Milestone Inc, CT) in a small subsection of wet brain tissue (whole brain or CER, CBL and OL) for each individual according to procedures outlined in Chapter 4. Due to the small quantity of brain tissue in day 19 embryos, THg was measured only in CER. Previous work has found that THg does not vary between brain regions (Chapter 4). Residue information is presented on a wet weight basis. A Standard Reference Material (SRM) (DOLT-4, National Research Council of Canada) was included to monitor accuracy and precision. Accuracy for all studies averaged 99.0% of certified values and average precision (% relative standard deviation) averaged 6.4%.

5.2.3 Histology

Histological analysis was performed in day 19 chicken embryos from vehicle control eggs (n=5) and eggs injected with 6.4 μg/g (n=7) MeHgCl at day 0. Embryos for this analysis were decapitated and heads were immediately placed in 10% formalin after gently puncturing the skull. Fixed brains were removed, embedded in paraffin, and sectioned, and slides of brain sections were stained with hematoxylin and eosin (H&E). Purkinje cell counts were performed on ten visual fields, which were then averaged, in the cerebellum of each individual. Data are presented as cells/ mm. All histological procedures and interpretations were performed according to standard procedures by trained technicians and pathologists at the

Unit for Laboratory Animal Medicine (ULAM) Pathology Cores for Animal Research (PCAR), University of Michigan.

5.2.4 Neurochemistry

Homogenate and membrane preparations were prepared as described previously (Rutkiewicz et al. 2011). Brains were homogenized in 50mM Tris buffer (50 mM Tris HCl, 50 mM Tris Base, pH 7.4) and an aliquot was saved for enzyme analyses. Membranes were isolated by centrifuging at 48000 x g for 15 minutes. The pellet was washed three times and resuspended in Tris buffer. Protein content was measured using the Bradford assay and samples were stored at -80°C. Enzyme activity was measured in homogenate from all treatment groups in Study 1 and Study 3, and from the control and 6.4 µg/g treatment group in Study 2. Enzyme activity was not measured in day 10 embryos in Study 3 due to limited tissue quantity. Activity of GS was determined in a colorimetric assay measuring the production of γ-glutamyl hydroxamate from glutamine, and activity of GAD was measured in a radioactive assay measuring the production of [14C] CO_2 from [14C] glutamate (Rutkiewicz et al. 2011). Binding to the NMDA and $GABA_A$ (benzodiazepine) receptors was determined by radioligand binding assay as previously described (Rutkiewicz et al. 2011). Receptor binding was measured in samples from all treatment groups in all studies, but was the $GABA_A$ (benzodiazepine) receptor was not measured in brain from day 10 embryos in Study 3 due to limited tissue quantity. Variability was monitored through the use of internal, pooled controls. For all studies, GS within day variability averaged 5.2 %

and between day variability averaged 8.4% GAD within day variability averaged 8.8% and between day variability averaged 14.2%. Between plate variability averaged 12.3% for the NMDA receptor and 8.8% for the GABA receptor. Between day variability averaged 8.8% for the NMDA receptor and 10.6% for the GABA receptor.

5.2.5 Behavior

Behavioral tests were performed on chicks at days 1 and/or 7 post-hatch. Chicks were housed together in a brooder until they were removed for behavioral testing. Chicks were individually removed and tested for righting response and on the level and angled balance beam, then they were returned to the brooder until they were removed in pairs and tested for startle response (auditory habituation). All observations were performed by the same set of researchers.

5.2.5.1 Righting Response:

A chick's ability to right itself was tested on one day old and one week old hatchlings according to the protocol of Burger et al. (1995a). Each chick was individually placed on its back and the time it took for the chick to return to a righted position was recorded. Chicks were allowed to remain on their back for a maximum of 2 minutes. For chicks that did not right themselves within this period, their time was recorded as 120 seconds.

5.2.5.2 Level Balance Beam:

Balance was tested by placing each chick on a level balance beam (6 cm x 35 cm wooden plank covered with fine grit sandpaper) and allowing the chick the opportunity to walk for up to 30 seconds (Burger et al. 1995a). Because one day old chicks did not willingly move on the beam, only 7 day old chicks were tested. If the chick moved, the length of time the chick remained on the balance beam as well as the distance the chick traveled were recorded.

5.2.5.3 Angled Balance Beam:

An angled balance beam test (Burger et al. 1995a) was also on one day old and one week old chicks. Chicks were placed on the end of a level balance beam (6 cm x 35 cm wooden plank covered with fine grit sandpaper attached by a hinge to a second wooden plank), and the beam was slowly raised at a rate of 3 degrees per second. The angle at which the chick first took a step as well as the angle at which the chick fell off the beam were recorded.

5.2.5.4 Startle Response:

Auditory habituation (startle response) was tested using a modification of the procedures of Rogers et al. (1974). Chicks were placed in pairs in 20 cm x 25 cm x 20 cm wooden boxes lined with paper and were allowed to move and peck freely. Every 30 seconds two polypropylene blocks were clapped together and the chicks were watched for a startle response (ex. flinching, heat lift, disruption from activity, etc.). The test was ended when a chick did not respond for two consecutive claps, and the number of the last startle response was recorded as the score.

5.2.6 Statistics

All statistics were performed using IBM SPSS Statistics (V.19.0, New York). Relationships between brain THg and neurochemical and behavioral endpoints were assessed using Spearman correlation. Kruskal-Wallis, followed by Mann-Whitney, tests were used to compare neurochemical and behavioral endpoints between dose groups. Logistic regression was used to determine if brain THg (continuous variable) or dose group (categorical variable) increased the odds of a chick taking longer than 15 seconds to right itself or the odds of a chick moving on or falling off of the level balance beam. Fisher's exact test was used to compare the proportion of brains with lesions between control and dosed embryos and the Mann-Whitney test was used to compare cerebellar Purkinje cell counts between control and dosed brains. The critical level of significance was set at 0.05 for all tests.

5.3 Results

5.3.1 Mortality Rates

Mortality rates varied between studies (Table 5.2). In chickens, mortality rates at all stages (embryonic and hatching) were generally low in both vehicle and non-inject control eggs and were higher in eggs injected with MeHgCl, but mortality did not increase in a dose dependent manner. Similarly in chicken eggs injected with MeHgCys, mortality was low in vehicle control eggs and higher in dosed eggs.

In Japanese quail embryos, mortality was higher in vehicle control eggs than in non-inject control eggs, and was highest in eggs injected with both doses of MeHgCl.

5.3.2 Brain THg Concentrations

A thorough report of brain and tissue residues in chicken embryos and hatchlings and quail embryos can be found in Chapter 4. Briefly, in chickens, brain Hg concentrations in control chicks were low, with averages between 0.01-0.04 μg/g at all embryonic and hatchling stages. Whole brain THg in chicks from eggs injected with 6.4 μg/g egg averaged 0.07 μg/g at day 10, 0.35 μg/g at day 14, and 2.13 μg/g at day 19. Cerebrum THg in chicks from eggs injected with 6.4 μg/g egg averaged 1.92 μg/g in day 19 embryos, 3.98 μg/g in day 1 hatchlings and 4.33 μg/g in day 7 hatchlings. Concentrations did not differ significantly between brain regions. In day 15 Japanese quail embryos, brain THg in embryos from vehicle control eggs and eggs injected with 6.4 μg/g egg averaged 0.015 μg/g and 1.84 μg/g, respectively. Whole brain THg in day 19 embryos from eggs injected with 6.4 μg/g MeHgCys averaged 0.91 μg/g.

5.3.3 Histology

Histological analysis revealed subtle lesions in the cerebellum of both control and dosed embryos (Fig. 5.1). Typical abnormalities included thickening of and variations in thickness of the external granule layer, displacement of Purkinje cells in the internal granule layer, paucity of Purkinje cells, and occasional pyknosis and

chromatolysis of Purkinje cells. Types of abnormalities observed were similar in control and dosed embryos. The proportion of embryos with brain lesions did not differ significantly between control (2/5 embryos) and dosed (3/7) embryos and may represent an artifact of sample processing. No abnormalities were identified in the spinal cord, cerebral cortex, brainstem, or medulla oblongata of any embryo. The mean ± SD Purkinje cell linear density was 38.7 ± 3.7 cells/mm in the cerebellum of control embryos and 39.8 ± 5.8 cells/mm in the cerebellum of dosed embryos. Purkinje cell linear density did not differ significantly between control and dosed embryos.

5.3.4 Neurochemistry

In all brain regions, THg concentrations in CER, CBL, and OL of day 19 embryos were not significantly correlated with GS or GAD activity or NMDA or $GABA_A$ (benzodiazepine) receptor levels (Fig. 52), and enzyme activity and receptor levels did not differ between dose groups. Similarly in day 1 hatchlings, enzyme activity and receptor levels did not correlate significantly with brain THg or differ by dose group in any brain region in either study. In day 7 hatchlings, GAD activity and NMDA receptor levels were not associated with brain THg in any brain region in either Study 3 or 4. In Study 3, THg was significantly positively associated with GS activity ($p=0.003$, $r_s=0.754$), and GS activity ($p=0.018$) and $GABA_A$ (benzodiazepine) receptor levels ($p=0.01$) were significantly higher in chicks from the 6.4 μg/g dose group compared to controls only in optic lobe. However, these findings were not reproduced in Study 4.

In whole brain of day 10 chicken embryos, whole brain THg was not associated with NMDA receptor levels and levels did not differ between dose group. In day 14 embryos, THg was not associated with GS or GAD activity or NMDA or $GABA_A$ (benzodiazepine) receptor levels in whole brain. In day 19 embryos, GAD activity was significantly positively associated with THg in whole brain ($p<0.001$, $r_s=0.600$), and activity was significantly higher in embryos from both groups dosed with MeHgCl than from control eggs (3.2 μg/g egg: p=0.031, 6.4 μg/g egg: p=0.021). Brain THg was not associated with GS activity or NMDA or $GABA_A$ (benzodiazepine) receptor levels in whole brain of day 19 embryos.

In day 19 chicken embryos injected with MeHgCys rather than MeHgCl, brain THg was positively associated with NMDA receptor levels ($p=0.023$, $r_s=0.601$) and negatively associated with $GABA_A$ (benzodiazepine) receptor levels ($p=0.021$, $r_s=-0.610$), but receptor levels did not differ significantly between dose groups. Activity of GS and GAD was not associated with brain THg and did not differ between dose groups (Fig. 5.3).

In whole brain of day 15 Japanese quail embryos, brain THg was not significantly correlated with enzyme activity or receptor levels. GAD activity in embryos from the 6.4 μg/g dose was significantly lower than in embryos from the control (pooled vehicle and non-inject) group (p=0.021). Neurochemical values for Japanese quail are presented in Table 5.3.

5.3.5 Behavior

5.3.5.1 Righting Response

Righting response time was not associated with THg in any brain region in day 1 chicken hatchlings, and did not differ between dose groups (Table 5.4). Additionally, neither THg nor dose group was associated with the odds of a righting time greater than 15 seconds. In day 7 hatchlings, righting time did not correlate with THg in any brain region. Mean righting time was significantly higher in chicks from the 3.2 μg/g dose group than in chicks from the control group (p=0.011) in Study 4 but did not differ between dose groups in Study 3. Brain THg and dose group were not associated with odds of having a righting time greater than 15 seconds in day 7 hatchlings in either study.

5.3.5.2 Level Balance Beam

The level balance beam test was only performed on day 7 hatchlings because day 1 hatchlings did not walk willingly on the balance beam. Odds of a chick walking on the beam were not associated with THg in any brain regions or with dose group. Of those that did walk on the beam, odds of falling off the beam and distance traveled on the beam were not associated with THg in any brain region or with dose group (Table 5.4).

5.3.5.3 Angled Balance Beam

For the angled balance beam, Angle 1, the angle at which the chick first took a step on the balance beam, was significantly positively correlated with Thg in the optic lobe (p=0.047, r_s=0.714), but no other brain region, of day 1 hatchlings in Study 3 (Table 5.4). This finding was not reproduced in Study 4. Angle 1 did not

differ between dose groups in day 1 hatchling in either study. Angle 2, the angle at which the chick fell off the balance beam, was not associated with THg in any brain region and did not differ by dose group in day 1 hatchlings in either study. In day 7 hatchlings in both Study 3 and Study 4, neither Angle 1 nor Angle 2 were associated with THg in any brain region and did not differ by dose group.

5.3.5.4 Startle Response

Auditory habituation was tested in day 1 and day 7 hatchlings. The number or claps until chicks were no longer startled did not correlate with THg concentration in any brain region, and did not differ between dose group in either day 1 or day 7 hatchlings (Table 5.4).

5.4 Discussion

This work is the first to address potential neurological impacts of developmental MeHg exposure in birds using a multi-tiered approach that considers effects at the neuropathological, neurochemical, and neurobehavioral levels. Developing embryos are often considered to be particularly sensitive to the neurological impacts of toxicants, with effects apparent at low doses due to the incomplete formation of the blood brain barrier (Grandjean 2006). Pathological lesions are one of the most widely reported changes associated with MeHg exposure. We examined brains of chicken embryos from eggs injected with 0 and 6.4 μg/g MeHgCl for histological abnormalities, and found that despite accumulating brain THg concentrations of approximately 2.0 μg/g by day 19, MeHg exposure was

not associated with lesions or Purkinje cell density in embryonic chicken brain. In several individuals abnormalities were detected in the cerebellum, which is a region shown to be impacted by MeHg in mallard chicks exposed to MeHg developmentally (Heinz et al. 1976) and in great egret chicks (Spalding et al. 2000), northern goshawk juveniles (Borg et al. 1970), and mallard (Pass et al. 1975) and American kestrel (Bennett et al. 2007) adults exposed through the diet. However, in our study these changes occurred equally in control and dosed embryos. It must be noted that in our studies, brain THg concentrations in day 19 embryos were lower than those seen in the chicks, juveniles, and adults that displayed lesions in the aforementioned studies. Our findings for Purkinje cell density are in contrast to those of Carvalho et al. (2008), who found that a single injection of 0.1 μg MeHgCl into egg yolk at embryonic day 5 resulted in significantly decreased numbers of Purkinje cells and increased distance between Purkinje cells in 5 day old hatchlings.

Neurochemical changes have recently been employed as markers of MeHg's effects. Changes have been seen in adult bald eagles and common loons exposed to dietary MeHg (Rutkiewicz et al. 2011, Scheuhammer et al. 2008), but effects of Hg exposure on neurochemistry in the developing avian brain have not been previously studied. One of the primary goals of this work was to identify Hg associated neurochemical changes in birds exposed *in ovo* to MeHg. We used a comprehensive approach, looking at neurochemistry in chickens during three embryonic and two post-hatch timepoints, in both chickens and Japanese quail, and in whole brain as well as three distinct brain regions. Though brain THg concentrations were similar to those in wild adult birds that displayed alterations (Rutkiewicz et al. 2011,

Scheuhammer et al. 2008), chicken embryos and hatchlings did not consistently display the Hg associated neurochemical changes seen in wildlife. These findings are somewhat unexpected, as changes with the four biomarkers investigated have been identified in several studies. The NMDA receptor has been consistently negatively associated with THg and inorganic mercury (IHg) in wild birds (Rutkiewicz et al. 2011, Scheuhammer et al. 2008), and with THg and MeHg in wild and laboratory mink (Basu et al. 2007a), and the $GABA_A$ (benzodiazepine) receptor has been positively associated with MeHg treatment in adult rats (Concas et al. 1983). Glutamine synthetase activity has been positively associated with brain THg and IHg in wild eagles (Rutkiewicz et al. 2011) and with MeHg exposure in adult rats (Kung et al. 1989), and GAD activity has been negatively associated with brain IHg in eagles (Rutkiewicz et al. 2011) and with postnatal MeHg treatment in rat pups (O'Kusky et al. 1988). Though we found a few statistically significant relationships between brain THg or dose group and neurochemistry, none of our findings were reproducible or consistent between studies.

We also assessed the impact of developmental Hg exposure on various behaviors in chicken hatchlings. Behavioral endpoints may be the most important to consider when trying to determine the potential for individual and population level effects of Hg, because many behaviors can impair survival and reproductive skills. Dietary Hg exposure has been linked to behavioral changes in several avian species in the laboratory (Bennett et al. 2007, Bouton et al. 1999, Kenow et al. 2010, 2011, Spalding et al. 2000). Effects of *in ovo* exposure on hatchling behavior are not well studied in birds, but Kenow et al. (2011) found that Hg exposure via egg

injection altered response to a frightening stimulus, but not righting response, in common loon hatchlings. Findings in rodent developmental toxicity studies vary, with some showing impairments in learning and other behaviors (Baraldi et al. 2002, Carratu et al. 2006), and some showing no effects (Cagiano et al. 1990, Carratu et al. 2006) on behavior in pups. We assessed several behaviors in one and seven day old chicken hatchlings. Righting times in our studies ranged from 0.9 s – 126 s, with the majority of chicks righting within 2 s. This is similar to righting times seen in common loon chicks (Kenow et al. 2010) and faster than those seen in herring gull chicks (Burger et al. 1995a. Unlike in herring gull chicks (Burger et al. 1995a, 1995b), the time before stepping or falling on the angled balance beam varied greatly for chicken hatchlings. Only one week old chicks moved willingly on the level balance beam, but of those that moved, distances were similar to those seen in herring gulls (1995a). Because just 59% of chicks moved on the beam, this test may not be ideal for chicken studies. Not surprisingly considering the lack of pathological and neurochemical alterations in all studies, we did not find evidence of MeHg associated behavioral changes for any of our tests.

The lack of MeHg associated pathological, neurochemical, and behavioral changes in our studies may be attributed to a variety of factors. Firstly, and perhaps most importantly, we must consider that the effects of developmental MeHg exposure in embryos and young hatchlings may differ from those in older birds with long-term dietary exposure. Though thorough comparisons of neurological effects of MeHg in chicks and adult birds has not been performed, we can draw from the extensive mammalian literature to infer that effects of MeHg may differ between life

stages. Prenatal exposure in mammals typically results in widespread brain damage, as opposed to damage in anatomically and functionally discrete brain regions as seen in adults (reviewed in Clarkson et al. 2006). Effects of MeHg differ between neonates and adults for GAD activity (Concas et al 1983, O'Kusky et al. 1988), and most likely for other neurochemical biomarkers as well. In one of the few studies of MeHg's behavioral impacts in young birds, dietary exposure impaired righting response, an endpoint that was not affected by *in ovo* exposure (Kenow et al. 2011), in older common loon chicks (Kenow et al. 2010). Often, MeHg's impacts do not become apparent until long after exposure. A latency period of up to several years after prenatal and early postnatal life MeHg exposure may occur before the onset of some neurobehavioral changes (Rice 1996, Spyker 1975). Additionally, impacts of developmental MeHg exposure are greatly enhanced with additional postnatal exposure (Rice 1998, Spyker 1975) in mammals. If this is true in birds as well, effects may be most apparent in adults with *in ovo* followed by years of dietary exposure, such as adult common loons with altered reproductive and chick rearing behaviors (Evers et al. 2008, Nocera et al. 1998). As illustrated by the slight differences with GAD activity in chickens and quail, neurological impacts of MeHg may differ between species as well. For example, brain lesion characteristic of MeHg toxicity in humans are not apparent in non-human primates (reviewed in Clarkson et al. 2006), and otters and mink display opposing correlations between MeHg and cholinesterase activity and muscarinic cholinergic receptor levels (Basu et al. 2005, 2006, 2007b). Heinz et al. (2009) have shown that embryo mortality due to MeHg differs between species, and neurological effects may differ as well.

Considering that we tested two gallinaceous species here, these studies should not be considered a thorough evaluation of MeHg's developmental effects on neurochemistry and behavior in all birds. Finally, it is important to consider that while convenient and controlled, injection of MeHgCl into an egg is an unnatural exposure, and resulting effects may differ from other exposures. In another study of young chickens, *in ovo* MeHg exposure negatively influenced learning (Hughes et al. 1976); however this study involved yolk sac injection which likely produces a high acute exposure very early in development. That results can vary by exposure type is especially apparent considering that our findings in day 19 embryos from eggs injected with MeHgCl differed from those injected with MeHgCys.

Collectively, these studies suggest that while embryos are generally considered to be sensitive to toxicants, *in ovo* exposure to MeHg may not be sufficient to cause neurochemical and behavioral alterations in embryos and hatchlings of at least some avian species. Therefore, long-term dietary exposure that impacts adult reproductive and chick rearing abilities may instead be a greater risk factor for some avian populations. Our findings do not support previous field studies demonstrating associations between Hg and neurochemical biomarkers, but rather draw attention to factors like age and species that may influence responses to MeHg. This work also evokes some questions about the suitability of egg injection techniques as a tool for studying neurochemical biomarkers in birds. These issues present potential limitations that must first be addressed if neurochemical biomarkers are to be useful for assessing Hg risks in wild birds. Future studies on

chronically exposed adult birds and their offspring, as well as egg injection studies on a variety of species may help to address these uncertainties.

Figures and Tables

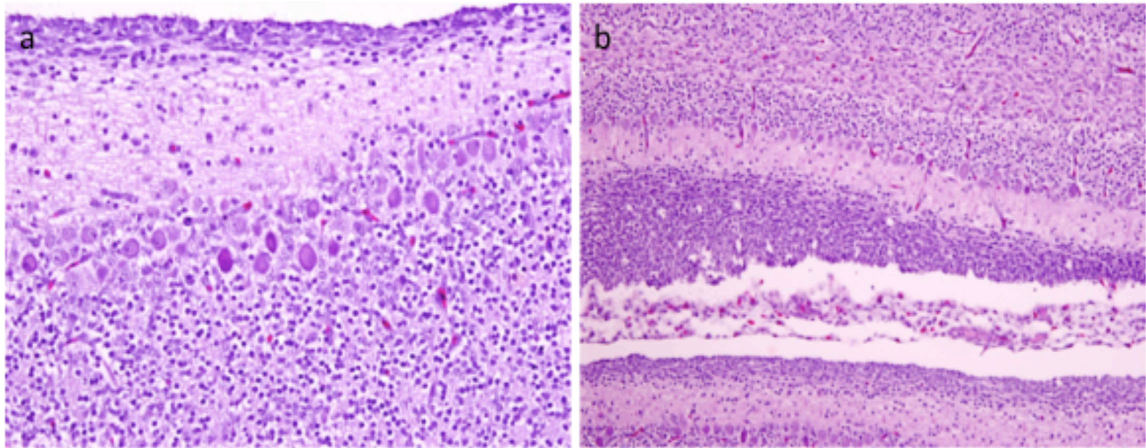

Figure 5.1 Neuropathology in cerebellum of day 19 chicken embryos. Sections of day 19 white leghorn chicken cerebellum demonstrating **a** Purkinje cell displacement and chromatolysis (H&E stain, 20X) and **b** variation in the thickness of the external granule layer (H&E stain, 10X) that was observed equally in eggs injected with vehicle control and 6.4 μg/g methylmercury chloride

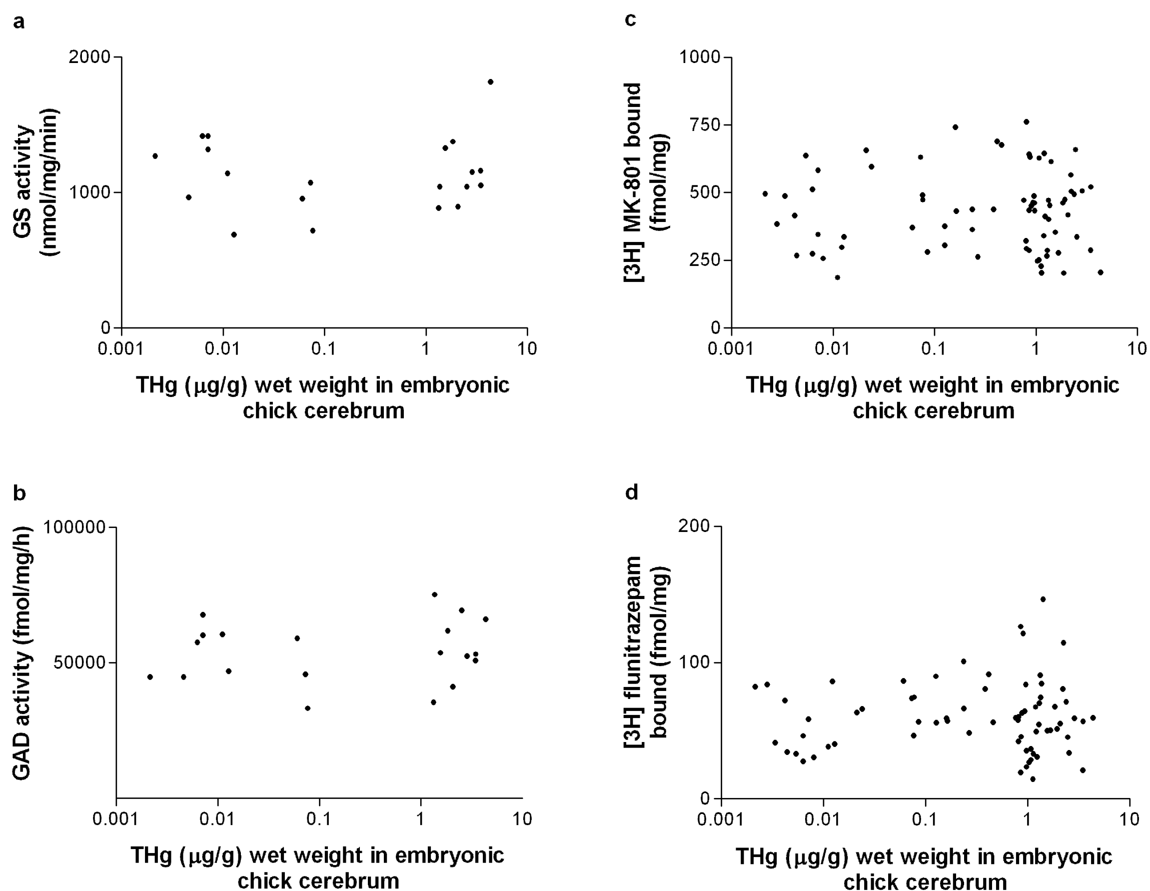

Figure 5.2 Brain mercury and neurochemistry in day 19 chicken embryos exposed to methylmercury chloride by egg injection. Associations between total mercury (THg) and **a** glutamine synthetase (GS) activity, (r_s=0.012, p=0.959, n=20), **b** glutamic acid decarboxylase (GAD) activity(r_s=0.152, p=0.523, n=20), **c** N-methyl-D-aspartic acid (NMDA) receptor levels (r_s=-0.008, p=0.949, n=73), and **d** γ-aminobutyric acid ($GABA_A$) receptor levels (r_s=-0.017, p=0.890, n=72) in cerebrum of day 19 chicken embryos from eggs injected with methylmercury chloride at incubation day 0. Data presented were derived from Study 1 (Table 5.1)

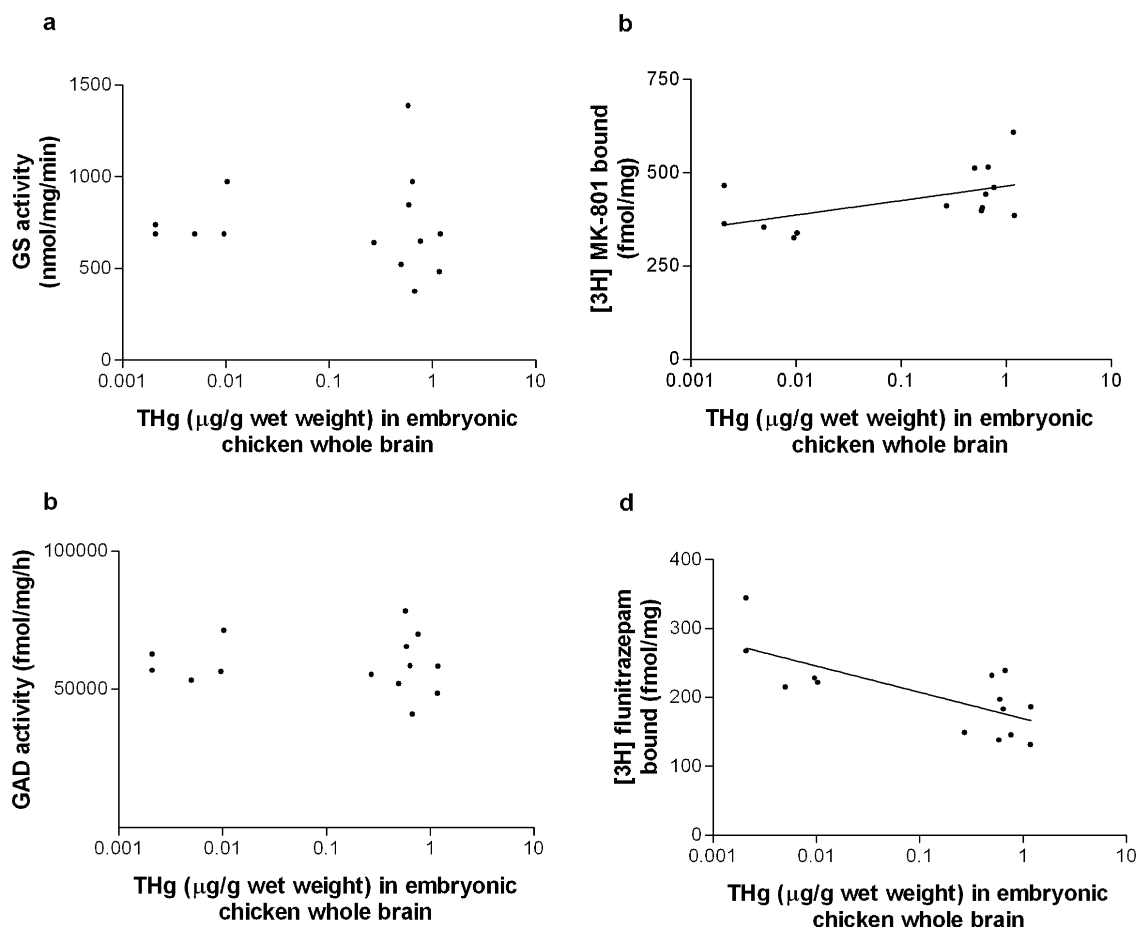

Figure 5.3 Brain mercury and neurochemistry in day 19 chicken embryos exposed to methylmercury cysteine by egg injection. Associations between total mercury (THg) and **a** glutamine synthetase (GS) activity, (r_s=-0.156, p=0.594, n=14), **b** glutamic acid decarboxylase (GAD) activity (r_s=-0.137, p=0.641, n=14), **c** N-methyl-D-aspartic acid (NMDA) receptor levels (r_s=0.601, p=0.023, n=14), and **d** γ-aminobutyric acid ($GABA_A$) receptor levels (r_s=-0.610, p=0.021, n=14)in cerebrum of day 19 chicken embryos from eggs injected with methylmercury cysteine at incubation day 0. Data presented were derived from Study 5 (Table 5.1)

Table 5.1 Experimental conditions and sampling details for mercury egg (air cell) injection studies

	Study 1	**Study 2**	**Study 3**	**Study 4**	**Study 5**	**Study 6**
Species	chicken	chicken	chicken	chicken	chicken	quail
Toxicant	MeHgCl	MeHgCl	MeHgCl	MeHgCl	MeHgCys	MeHgCl
Doses (µg/g egg)	0, 0.62, 2.0, 3.2, 6.4, non-inject	0, 3.2, 6.4	0, 6.4	0, 3.2, 6.4	0, 3.2, 6.4	0, 3.2, 6.4, non-inject
Sampling timepoints (embryonic or post-hatch day)	19	10, 14, 19	post-hatch 1, post-hatch 7	post-hatch 1, post-hatch 7	19	15
Brain regions sampled	CER, CBL, OL	whole brain	CER, CBL, OL	CER, CBL, OL	whole brain	whole brain
Neurochemical assays performed	NMDA receptor, GABA receptor, GS, GAD	NMDA receptor, GABA receptor, GS, GAD	NMDA receptor, GABA receptor, GS, GAD	NMDA receptor, GABA receptor, GS, GAD (post-hatch 7)	NMDA receptor, GABA receptor, GS, GAD	NMDA receptor, GABA receptor, GS, GAD
Behavioral tests performed			righting response, angled balance beam	righting response level balance beam, angled balance beam, , startle		

Table 5.2 Mortality rates in white leghorn chicken and Japanese quail embryos in mercury egg (air cell) injection studies. Sample sizes range from 6 to 22 eggs per dose group

		1	**2**			**3**	**4**	**5**	**6**
Timepoint		**d19**	**d10**	**d14**	**d19**	**hatch**	**hatch**	**d19**	**d15**
Dose group	**Non-inject**	0%							17%
	0 μg/g	0%	18%	9%	0%	14%	42%	0%	33%
	0.62 μg/g	17%							
	2.0 μg/g	21%							
	3.2 μg/g	11%	8%	0%	9%		36%	29%	42%
	6.4 μg/g	23%	8%	31%	31%	19%	64%	20%	42%

Table 5.3 Neurochemical receptor and enzyme values in Japanese quail embryos. Values (mean ± SD) for glutamine synthetase (GS) activity, glutamic acid decarboxylase (GAD) activity, N-methyl-D-aspartic acid (NMDA) receptor levels, and γ-aminobutyric acid ($GABA_A$) receptor levels in whole brain of day 15 Japanese quail embryos from eggs injected with methylmercury chloride at incubation day 0. Sample sizes are in parenthesis. Asterisk indicates dose group mean significantly different from control mean

	GS (nmol/mg/min)	GAD (pmol/mg/h)	NMDA (fmol/mg)	GABA (fmol/mg)
Control [a]	797.2 ± 121.7 (8)	139.4 ± 29.4 (8)	408.3 ± 120.5 (14)	133.4 ± 48.4 (14)
3.2 µg/g	738.3 ± 155.2 (7)	122.5 ± 30.6 (7)	408.3 ± 91.3 (7)	115.1 ± 53.4 (7)
6.4 µg/g	690.6 ± 196.1 (7)	95.7 ± 13.3 (7) *	439.8 ± 68.7 (7)	120.2 ± 53.7 (7)

[a] Vehicle and non-inject control samples did not differ significantly for brain total mercury or neurochemical values, and were therefore pooled for computation of descriptive statistics

Table 5.4 Behavioral tests in chicken hatchlings. Values (mean ± SD) for behavioral tests in one day old and seven day old white leghorn chicken hatchlings from eggs injected with methylmercury chloride at incubation day 0. Sample sizes are in parenthesis. Data presented were derived from Study 3/Study 4 (Table 5.1)

		DAY 1			**DAY 7**		
		0 μg/g	**3.2 μg/g**	**6.4 μg/g**	**0 μg/g**	**3.2 μg/g**	**6.4 μg/g**
Righting time (seconds)		5.6 ± 8.7 (6)/ 1.3 ± 0.6 (7)	NA/ 1.2 ± 0.4 (9)	33.3 ± 49.4 (5)/ 1.1 ± 0.2 (5)	1.1 ± 0.2 (5)/ 0.7 ± 0.1 (7)	NA/ 0.9 ± 0.1 (9)	24.7 ±40.6 (7)/ 0.7 ± 0.2 (5)
Level beam (cm moved)					NA/ 6.0 ± 5.6 (3)	NA/ 4.3 ± 3.0 (4)	NA/ 1.7 ± 0.6 (3)
Angled beam	**Angle 1**	24.3 ± 19.2 (6)/ 46.1 8.5 (7)	NA/ 44.1 ± 8.7 (9)	36.8 ±11.6 (4)/ 32.8 ± 11.5 (5)	41.4 ± 7.1 (5)/ 33.1 ± 14.2 (7)	NA/ 37.4 ± 13.0 (8)	47.2 ± 12.8 (6)/ 25.6 ± 12.4 (5)
	Angle 2	55.5 ± 10.6 (6)/ 59.3 ± 7.1 (7)	NA/ 61.3 ± 7.5 (9)	60.0 ± 6.3 (5)/ 64.4 ± 7.9 (5)	63.8 ± 11.4 (5)/ 65.1 ± 6.9 (7)	NA/ 66.0 ± 14.0 (8)	55.9 ± 10.7 (7)/ 63.6 ± 10.9 (5)
Startle response (number of claps)		NA/ 8.8 ± 5.0 (6)	NA/ 6.9 ± 4.6 (9)	NA/ 5.8 ± 3.2 (5)	NA/ 5.3 ± 5.6 (7)	NA/ 5.7 ± 3.8 (9)	NA/ 6.4 ± 2.8 (5)

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Chapter 6

Associations between brain mercury levels and neurochemistry in the thick-billed murre (*Uria lomvia*) embryo following methylmercury chloride egg injection

6.1 Introduction

Mercury (Hg) is a toxic metal that has become of increasing environmental concern in the Arctic (Dietz et al. 2011). Though very few point sources for Hg release exist in this region, several biogeochemical factors have resulted in the Arctic becoming a sink for Hg released elsewhere (Ariya et al. 2004). Due to it's long life in the atmosphere, elemental Hg may be transported from lower latitudes before it becomes oxidized and is deposited to the Arctic ecosystem in a phenomenon that is greatly augmented during annual spring ozone depletion events (Steffen et al. 2007). Biomethylation occurs via several proposed mechanisms in this cold climate (Barkay et al. 2011), consequently allowing for bioaccumulation of toxic methylmercury (MeHg) in fish and subsequent biomagnification in high trophic level wildlife (Jaeger et al. 2009). Mercury has been detected in tissues from several Arctic species, including fish Evans et al. 2005), mammals (Riget et al. 2004), and birds (Braune et al. 2006, Braune 2007). Recent studies have indicated rising concentrations Hg in wildlife tissues across the Arctic region (Braune 2007, Riget et al. 2011), therefore raising concerns about its potential toxicity in exposed species.

Mercury is an established neurotoxicant (Clarkson et al. 2006) that has been associated with clinical changes including brain lesions (Heinz et al. 1975, Spalding et al. 2000), and alterations in behaviors and motor skills (Evers et al. 2008, Kenow et al. 2010, Spalding et al. 2000) in birds both in the field and laboratory. Prior to inducing overt toxicity, Hg may be associated with alterations in receptors and enzymes involved in neurotransmission. These neurochemical biomarkers relate to brain function and changes may represent the earliest signs that Hg is impacting the brain (Manzo et al. 1996). Associations between brain Hg and such biomarkers have been identified in wild common loons (Scheuhammer et al. 2008) and bald eagles (Scheuhammer et al. 2008, Rutkiewicz et al. 2011) in relation to substantial Hg exposures.

The current study explores whether developmental MeHg exposure is associated with subclinical neurochemical changes in thick-billed murre (*Uria lomvia*) embryos in order to better understand potential risks for neurotoxic effects in this species. The thick billed murre is commonly found across polar regions and is known to be exposed to Hg in the Canadian Arctic (Braune et al. 2007). Though Hg concentrations in murre eggs are currently below predicted toxicity thresholds for birds, concentrations are believed to be on an upward trend (Braune et al. 2007). Because toxic responses may vary between species (Heinz et al. 2009), it is important to evaluate and understand the potential for toxicity in at-risk species. The objective of this study was to assess relationships between brain THg concentrations and levels of the *N*-methyl-D-aspartic acid (NMDA) and g-aminobutyric acid ($GABA_A$(benzodiazepine)) receptors and activity of glutamic acid

decarboxylase (GAD) and glutamine synthetase (GS) in thick-billed murre embryos following *in ovo* exposure to MeHg. This work will help to determine if this species may be susceptible to neurological changes if levels of Hg continue to rise in the Arctic.

6.2 Methods

6.2.1 Animals

Tissues were provided by Birgit Braune (Environment Canada) and were a subset of samples form Environment Canada's larger study of the toxicity of Hg in thick-billed murre embryos. Details of the experimental design can be found in Braune et al. (2012). Fertilized thick-billed murre eggs were collected soon after laying from a colony on Coats Island, NU, and were flown soon after to the National Wildlife Research Centre (NWRC) in Ottawa, ON. After incubating eggs in a Brinsea Z6 incubator for 5 days, eggs were drilled and injected with methylmercury chloride according to a procedure modified from Heinz et al (2009). Eggs from Coats Island contained a mean of 0.2 μg/g wet weight THg and were injected with 0 (vehicle control), 0.05, 0.1, 0.2, 0.4, 0.8, 1.6, 3.2 μg/g wet weight methylmercury chloride (MeHgCl) dissolved in safflower oil. A non-inject control was also included. Chicks were dissected at pipping (approximately 33 days) and brains were removed, homogenized by stirring with a spatula in a cryovial, and frozen in liquid nitrogen. A subsample of approximately 0.2 g of homogenized whole brain from a subset of 51 embryos was provided to us for this analysis.

6.2.2 Hg Analysis

Total mecury (THg) in whole brain was measured as part of Environment Canada's study, and values were provided for neurochemical analysis. Briefly, THg in freeze dried tissue was measured according to the standardized protocol (NWRC Method No. MET-CHEM-AA-03H) described by Braune et al. (2012). Intermittent blanks, duplicate samples, and the standard reference materials (SRMs) OT1566b (National Institute of Standards and Technology), TORT-2, and DOLT-4 (National Research Council of Canada) were used to monitor accuracy and precision. Average accuracy was 107% of certified values, and average precision (% relative standard deviation) was 3%. Values are reported on a dry weight basis.

6.2.3 Neurochemical Assays

Neurochemical assays for the NMDA receptor, $GABA_A$(benzodiazepine) receptor, GAD, and GS were performed in whole brain according to the methods of Rutkiewicz et al. (2011) with few adaptations. GS activity was measured in homogenate rather than supernatant. For all assays, a pooled control sample was used to monitor variability. Between plate variability was 14% for the NMDA receptor and 16% for the $GABA_A$ (benzodiazepine) receptor. Between day variability was 8% for GS and 5% for GAD.

6.2.4 Statistics

Statistics were analyzed using IBM SPSS Statistics (V.19.0, New York). Spearman correlation was used to assess the relationship between dose group and

brain THg, and between brain THg and neurochemical receptors and enzymes. Embryos were also divided into quartiles based on brain THg levels, and neurochemical receptors and enzymes were compared between quartiles using Kruskal-Wallis and Mann-Whitney tests. For all analyses, p-values below 0.05 were considered to be significant.

6.3 Results

6.3.1 Hg Analysis

Brain THg concentrations increased significantly with dose group (r_S=0.782, p<0.001, n=51). Brain THg in non-inject control embryos averaged 0.90 µg/g (SD: 0.59, range:0.43-2.17 µg/g) and in vehicle control embryos averaged 0.82 µg/g (SD: 0.29, range:0.39-1.27. Across all dose groups, total Hg concentrations in the brain spanned a wide range from 0.34 µg/g to 10.77 µg/g with a mean of 2.16 µg/g. Concentrations for each dose group are presented in Table 6.1.

6.3.2 Neurochemical Assays

Overall, levels of the NMDA and $GABA_A$ receptors (reported as fmol radioligand bound per mg protein) averaged 399.3 fmol/mg (range: 231.3-691.3) and 114.6 fmol/mg (range: 51.1-259.7), respectively. Activity of GS (reported as nmol γ-glutamyl hydroxamate produced per mg protein per minute) averaged 1634.9 nmol/mg/min (range: 679.4-2534.9). Activity of GAD (reported as pmol CO_2 produced per mg protein per hour) averaged 128.1 pmol/mg/h (range: 14.6-237.5). Neurochemical values for each dose group are presented in Table 6.2.

Brain THg was positively correlated with levels of the NMDA receptor (r_s=0.439, *p*=0.001, n=51) and negatively correlated with activity of the enzyme GAD (r_s=-0.286, *p*=0.044, n=50) (Fig. 6.1). There were no significant associations between brain THg and the $GABA_A$ (benzodiazepine) receptor (r_s=-0.017 , *p*=0.909, n=50) or the enzyme GS (r_s=-0.053 *p*=0.711, n=51). Significant differences between quartiles for brain THg were detected only for the NMDA receptor. For this biomarker, receptor levels in embryos from the highest quartile for brain THg (brain THg>2.6 µg/g) were significantly higher than in embryos from each of the first three quartiles ($p \leq 0.006$).

6.4 Discussion

The thick-billed murre is increasingly becoming exposed to Hg in the Canadian Arctic, but little is known about the potential for Hg related neurotoxic effects in this species. In this study we assessed the relationships between brain Hg and four neurochemical receptors and enzymes in order to better understand what level of exposure might lead to neurological impacts. We found that brain THg was positively correlated with NMDA receptor levels and negatively correlated with GAD activity, but not related to $GABA_A$ (benzodiazepine) receptor levels and GS activity. Our findings in murre embryos differ from our findings in Japanese quail embryos and white leghorn chicken embryos and hatchlings (Chapter 5), which did not display significant relationships between brain THg and neurochemical receptors and enzymes. Another study of the larger set from which these murre samples are a subset also found no significant relationship with the NMDA receptor (Braune et al.

2011). Our findings also differ from those seen in wild bald eagles and common loons, which did exhibit similar variability and an overall negative association between Hg (in the form of IHg) and GAD activity, but also exhibited a positive association between THg and GS activity and a negative relationship between THg and NMDA receptor levels (Rutkiewicz et al. 2011, Scheuhammer et al. 2008). These observed differences may be attributed to a variety of factors including species differences, experimental design, animal age, and type of exposure.

It is clear that avian species respond differently to MeHgCl exposure via egg injection, and this must be considered as a possible explanation for contradictory responses in different species. In a study of 23 species, Heinz et al. (2009) found that LC_{50}s ranged from less than 0.25 μg/g to over 2.0 μg/g, demonstrating the great variability of responses that may occur following exposure. Additionally, distribution and metabolism of Hg, as well as pathological and neurochemical responses to Hg differ between mammalian species (reviewed in Basu et al. 2010). Similar differences in sequestration and demethylation of Hg have been identified in birds (Scheuhammer et al. 2008), and differences in neurochemical responses may vary as well. Though some neurochemical changes are likely due to direct effects of Hg on receptors and enzymes, others are thought to be secondary, adaptive changes to maintain homeostasis following the primary insult. It is possible that the murre, a fish eating wild species, evolved to make such adaptations whereas the chicken, a domesticated grain and insect eating species, has not.

Experimental design must also be considered when interpreting findings from this study and those described in Chapter 5. While all embryo studies followed

similar egg injection methods, the chicken and quail studies involved injecting eggs at day 0, while the current study involved eggs that were injected after five days of incubation, which is approximately equivalent to three days of incubation in a chicken, as in Heinz et al. (2009). Heinz et al. (2006) have found that embryo mortality rates differ depending on the day of MeHgCl injection. When eggs are injected at day 0, mortality rates are quite low and variable at all treatment levels. When injected at later timepoints, mortality rates are greatly increased and display a clear dose response relationship. This apparent onset of embryotoxicity only with later injections suggests that the embryos receiving exposure starting at day 0 may not display the same physiological changes, such as those in neurochemical biomarkers, as if exposure had commenced after initial incubation. However, it is important to consider that the exposure scenario in this murre study is less realistic than exposure prior to incubation and may not represent effects in embryos that are exposed to Hg through maternal deposition and are incubated naturally in the field. Another consideration to make is that the eggs used in this study were collected from the wild, and likely contained a variety of environmental contaminants that potentially influenced neurochemistry and could have interfered with out findings. Nevertheless, this work does suggest that Hg may relate to some neurochemical changes in thick-billed murre brain tissues.

In addition to differering from responses in chicken and quail embryos, responses in murres differed from those in wild bald eagles and common loons. It is very likely that effects of developmental exposure in an embryo differ from effects of dietary exposure in an older animal. Because neurotransmitters play important

roles in embryonic brain development (Herlenius et al. 2004), and the role of specific neurotransmitters in developing organisms can differ from that in adults (Ben-Ari et al. 1997), it is unsurprising that direct and adaptive responses in embryos and adults may differ. Our most perplexing finding here was that although we found that the NMDA receptor was negatively associated with Hg in wild birds (Rutkiewicz et al. 2011, Scheuhammer et al. 2008) it was positively associated with Hg in murre embryos. Similar increases in NMDA receptor levels have occasionally been observed with developmental exposure to other chemicals. Prenatal exposure to the developmental toxicant valproic acid increases NMDA receptor levels in rats (Rinaldi et al. 2007), and developmental exposure to lead has also increased binding of [3H] MK-801 binding in rat brain in some cases, depending on the age at which effects on the NMDA receptor were studied (Jett et al. 1995). The NMDA receptor plays important roles in avian brain development (Andaloro et al. 1998), and Hg is known to inhibit direct binding of [3H] MK-801 to the NMDA receptor in some neonatal and adult rats (Rajanna et al. 1997). Perhaps an increase in NMDA receptor levels could represent a compensatory response to ensure enough functional receptors to fulfill its developmental role. However, we cannot exclude the possibility of Type 1 statistical error here, especially considering that Braune et al. (2012) found no relationship between Hg and the NMDA receptor in a larger sample of murre brains from the same study and we have seen no such association in chicken or quail embryos (Chapter 5). We did find a reduction in GAD activity similar to that which we saw in adult bald eagles. This could reflect a direct inhibition of GAD by Hg in the embryo, rather than an adaptive change. One

biomarker, GS, was positively associated with Hg in wild adult birds (Rutkiewicz et al. 2011) but not associated with Hg in murre embryos. Methylmercury is known as a toxicant with a period of latency between exposure and toxicity (Clarkson et al. 2006), which may account for this difference.

The overall objective of this work was to determine if the thick-billed murre may be at risk for neurotoxicity as Hg levels rise in the Canadian Arctic. In this controlled laboratory study, we found some evidence of Hg-associated changes in the NMDA receptor and GAD enzyme. Quartile analysis suggests that changes in NMDA receptor levels may be significant in individuals with the highest levels of brain THg, although as mentioned previously, this relationship may be spurious as it was not reproduced in a larger set of murre embryos thus may not truly indicate a MeHg induced change. Activity of GAD, though correlated with brain THg, was very variable at all exposure levels and was not significantly reduced in even the highest quartile. The other biomarkers studied were not associated with brain THg. These findings suggest that thick-billed murres may be at risk for some neurochemical alterations if Hg levels continue to rise substantially, but current exposures are not likely to pose a threat.

Figures and Tables

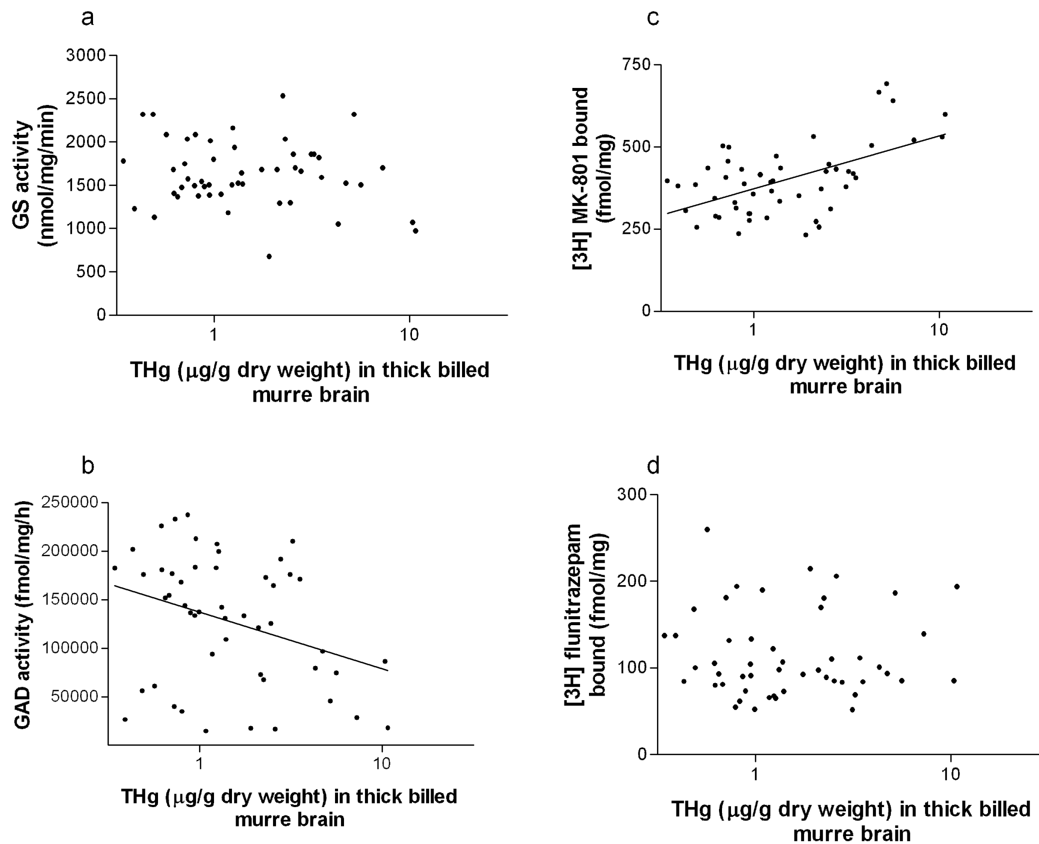

Figure 6.1 Brain mercury and neurochemistry in pipping thick-billed murre embryos exposed to methylmercuy chloride by egg injection. Associations between total mercury (THg) and (a) glutamine synthetase (GS) activity, (r_s=-0.053 *p*=0.711, n=51), (b) glutamic acid decarboxylase (GAD) activity (r_s=-0.286, *p*=0.044, n=50), (c) N-methyl-D-aspartic acid (NMDA) receptor levels (r_s=0. 439, *p*=0.001, n=51), and (d) g-aminobutyric acid ($GABA_A$) benzodiazepine receptor levels (r_s=-0.017 , *p*=0.909, n=50) in the brain of thick-billed murre pipping embryos from eggs injected with methylmercury chloride at incubation day 3. Lines represent best-fit linear regression

Table 6.1 Mercury in thick-billed murre pipping embryo brain. Mean total mercury concentrations (mean ± SD) in whole brain of thick billed murre pipping embryos from eggs injected with methylmercury chloride on incubation day 5

Dose (µg/g)	**Mean (µg/g)**	**Range (µg/g)**	**n**
Non-inject	0.90±0.59	0.43-2.17	7
Vehicle	0.82±0.29	0.39-1.27	10
0.05	0.97±0.68	0.34-2.24	6
0.1	1.19±0.39	0.71-1.91	8
0.2	2.28±0.67	1.33-3.44	7
0.4	2.67±1.01	0.73-3.55	6
0.8	5.43±1.15	4.31-7.30	5
1.6	10.77±N/A	N/A	1
3.2	10.38±N/A	N/A	1

Table 6.2 Neurochemical receptor and enzyme values in thick-billed murre pipping embryos. Values (mean ± SD) for glutamine synthetase (GS) activity, glutamic acid decarboxylase (GAD) activity, N-methyl-D-aspartic acid (NMDA) receptor levels, and γ-aminobutyric acid ($GABA_A$) receptor levels in whole brain of pipping thick billed murre embryos from eggs injected with methylmercury chloride at incubation day 0. Sample sizes are in parenthesis

	GS (nmol/mg/min)	**GAD (pmol/mg/h)**	**NMDA (fmol/mg)**	**GABA (fmol/mg)**
Non-inject	1545.6 ± 397.5 (7)	154.5 ± 42.1 (7)	292.4 ± 42.2 (7)	86.1 ± 40.9 (7)
Vehicle	1646.4 ± 335.0 (10)	158.9 ± 71.2 (10)	386.5 ± 77.9 (10)	112.5 ± 31.2 (10)
0.05 μg/g	1810.1 ± 440.2 (6)	122.6 ± 85.5 (6)	353.5 ± 74.6 (6)	160.1 ± 62.5 (6)
0.1 μg/g	1561.8 ± 479.0 (8)	113.2 ± 64.9 (8)	344.7 ± 67.9 (8)	122.0 ± 63.7 (8)
0.2 μg/g	1676.6 ± 229.3 (7)	118.7 ± 53.2 (6)	411.7 ± 74.7 (7)	114.9 ± 41.0 (6)
0.4 μg/g	1810.1 ± 159.1 (6)	158.9 ± 60.6 (6)	424.0 ± 28.3 (6)	74.5 ± 14.5 (6)
0.8 μg/g	1619.9 ± 458.2 (5)	64.9 ± 24.5 (5)	604.3 ± 86.2 (5)	121.0 ± 42.0 (5)
1.6 μg/g	973.9 ± N/A (1)	18.0 ± N/A (1)	598.4 ± N/A (1)	193.8 ± N/A (1)

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Chapter 7

Brain mercury and neurochemistry in young zebra finches (*Taeniopygia guttata*) exposed developmentally to methylmercury

7.1 Introduction

Mercury (Hg) is a well established neurotoxicant, and exposure to methylmercury (MeHg) via fish consumption has been associated with reproductive impairment (Burgess et al. 2008) and behavioral changes (Evers et al. 2008) in wild adult piscivorous birds. Laboratory studies have also demonstrated MeHg associated reproductive impairment (Albers et al. 2007), behavioral changes (Bennett et al. 2007, Bouton et al. 1999, Spalding et al. 2000), and brain lesions (Bennett et al. 2007, Spalding et al. 2000) in juvenile and adult laboratory animals. Perhaps among the most sensitive markers of the neurological effects of Hg exposure are subtle changes in the receptors and enzymes that mediate neurotransmission. These changes in neurochemical biomarkers represent the earliest neurological changes and my help warn of Hg's effects before progression to clinical toxicity (Manzo et al. 1996). Associations between brain Hg levels and biomarkers involved in glutamate, GABA, and cholinergic neurotransmission have been identified in juvenile and adult wild bald eagles and common loons (Rutkiewicz et al. 2011, Scheuhammer et al. 2008). However, these neurochemical changes have not been reproduced in laboratory studies of embryonic and hatchling white leghorn chickens, Japanese quail, and thick-billed murres exposed to MeHg *in*

ovo (Chapter 5, 6), which suggests that the source and timing of exposure (i.e., developmental or dietary) may influence the neurological response.

Many recent studies of Hg exposure and effects in birds have shifted attention away from fish eating species to also focus on songbirds as awareness of impacts on terrestrial biota has grown. Cristol et al. (2008) demonstrated that many songbirds have elevated levels of blood Hg due to a diet rich in spiders and other insects with high concentrations of MeHg. Elevated levels of Hg have been detected in several terrestrial avian species in tidal marshes (Lane et al. 2011), along river shoreline downstream from a point source, and at study sites near a non-ferrous smelter (Janssens et al. 2003). Songbirds with high levels of Hg exposure have exhibited immunosuppression (Hawley et al. 2009), decreased reproductive success (Jackson et al. 2011), altered singing behavior (Hallinger et al. 2010), and reduced body condition (Janssens et al. 2003), thus demonstrating that Hg exposure may have damaging health impacts in terrestrial birds. Though Hg is primarily considered to be a neurotoxicant, no studies to date have addressed the effects of Hg on the songbird brain. These types of studies can be difficult to perform on wild birds, but studies on model organisms in the laboratory may help to predict potential effects of exposure in wild songbirds.

The goal of this study was to explore for potential neurological impacts of developmental and early life MeHg exposure in a model songbird, the zebra finch. We assessed levels of the NMDA and $GABA_A$ (benzodiazepine) receptors and the GS and GAD enzymes in juvenile zebra finches that were exposed to MeHg *in ovo*. These are four glutamatergic and GABAergic neurochemical that have previously

been studied in juvenile and adult bald eagles in the field (Rutkiewicz et al. 2011) and embryonic and hatchling chickens, quail (Chapter 5), and murres (Chapter 6) in the laboratory. Because this study involved the offspring of females who were fed MeHg, the chicks studied were exposed to maternally deposited MeHg from the time of fertilization throughout embryonic development. Unlike in the aforementioned egg injection studies on embryos and hatchlings (Chapter 5, 6), these birds experienced natural exposure to a biologically relevant form of MeHg (versus exogenous injection of a MeHg salt on embryonic day 0) during development and for several weeks posthatch, and were not assayed until several weeks after the onset of exposure. This work will increase our knowledge of the potential for subtle neurochemical effects from developmental MeHg exposure in songbirds, and complement our egg injection work by expanding our focus to a previously unstudied species and a study design that better represents a real world exposure scenario.

7.2 Methods

7.2.1 Animal Care

Animals were part of a study in the laboratory of Daniel Cristol (College of William & Mary, Williamsburg, VA, USA) and brains were kindly donated for our neurochemistry work. Birds used in this study were offspring of female zebra finches that were fed a methylmercury cysteine (MeHgCys) treated diet. Breeding birds were maintained in small (60”X30”X24”) wire cages in single sex groups until 10 weeks after start of dosing and in breeding pairs thereafter. Animal

rooms were maintained on a 14:10 hour light:dark cycle at ~20°C. Birds were provided with food and water ad libitum as well as access to grit and cuttlebone. A liquid vitamin supplement (Vita-Sol UltraVite Bird Vitamin Supplement) was added to the water supply to encourage breeding.

7.2.2 Food Preparation

Birds were fed a commercially available pelletized finch food (Zupreem FruitBlend Flavor Premium Daily Bird Food XS). Food was dosed by adding an aqueous solution of MeHgCys equal to 10% of the weight of the food to be dosed. Food was prepared in three mercury concentrations, 0.3, 0.6, and 1.2 μg/g wet weight. The control dose (0.0 μg/g) contained no mercury but a aqueous solution of cysteine equal to 10% of the weight of the food was added. After through mixing of the food and aqueous solution, actual mercury concentrations of each batch were measured from 10 subsamples of food. Mercury analysis was performed using atomic absorbance spectrometry on a Milestone DMA-80 direct mercury analyzer (Shelton, CT, USA). The average concentration of the subsamples was calculated to confirm that the concentration of the batch did not differ from the target concentration by more than 10%. All food was stored in freezers until being fed to birds.

7.2.3 Dosing

Adult birds (~100-300 days old) were randomly assigned to the four treatments (0.0, 0.3, 0.6, 1.2 μg/g dietary mercury) with 36 birds (18 males, 18

females) in each treatment. Birds were provided with dosed food only from the onset of dosing until the end of the study. For the first 10 weeks of dosing birds were kept in single sex cages with 3 or 4 birds in each cage. At 10 weeks the blood mercury levels appeared to have plateaued and the birds were randomly paired within treatment groups avoiding inbreeding. Each pair was kept in a separate cage visually isolated from other birds and provided with a nesting box and nesting material (hay) ad libitum. Boxes were monitored daily to record reproductive effort and hatching dates. Pairs were allowed to raise offspring until independence (50 days post hatching). All offspring were exposed the treatment continuously from the egg to independence through the parental diet as well as their own diet.

7.2.4 Tissue Collection

Offspring were sacrificed by rapid decapitation when the eldest chick in the brood reached 50 days post hatching. The brains were quickly removed from the skull and flash frozen in liquid nitrogen. Brains were stored at -80°C until processing. A gender matched subset of 96 brains was provided to us for this study. Both males (n=48) and females (n=48) were included in this analysis.

7.2.5 Mercury Analysis

Total mercury (THg) was measured in 5-15 mg subsections of cerebrum before the remainder of the brain was homogenized for neurochemistry. Concentrations were measured by thermal decomposition, amalgamation, and atomic absorption in a Direct Mercury Analyzer (DMA-80, Milestone Inc, CT) (EPA

method 7473; USEPA 1998). Concentrations are presented on a wet weight (w.w.) basis. The standard reference material DOLT-4 (National Research Council of Canada) was used to monitor accuracy and precision. Mean accuracy was 103% of certified values, and average precision (% relative standard deviation) was 3.5%.

7.2.6 Neurochemistry

Homogenate and membranes were prepared from whole brains according to protocols described previously (Rutkiewicz et al. 2011). Samples were stored at -20°C until neurochemical analysis. Activity of GS and GAD were measured in homogenate, and [3H] MK-801 binding to the NMDA receptor and [3H] flunitrazepam binding to the $GABA_A$ (benzodiazepine) receptor were measured according to published protocols (Rutkiewicz et al. 2011). Pooled homogenate and membrane were used as an internal controls for all sets of assays to monitor precision, which is reported as % relative standard deviation . Between set variability was 8% for GS and 18% for GAD. Between plate variability was 20% for the NMDA receptor and 17% for the $GABA_A$ (benzodiazepine) receptor.

7.2.7 Statistics

IBM SPSS (V.19.0, New York) was used for all statistical analyses. Spearman correlation was used to assess relationships between dose group and brain THg, between brain THg and neurochemical receptors and enzymes, and between chick age and neurochemical receptors and enzymes. Multiple linear regression was used to determine if the relationship between brain THg and neurochemistry changed

when age and gender were included as covariables. The Kruskal-Wallis test, followed by the Mann-Whitney test when necessary, was used to test for differences in brain THg concentration and neurochemical receptors and enzymes between dose groups. Mann-Whitney was used to compare neurochemical biomarker values between males and females. A p-value below 0.05 was considered to be significant for all tests.

7.3 Results

7.3.1 Brain THg Concentrations

Overall, THg concentrations in cerebrum ranged from 0.006 μg/g-7.491 μg/g. Concentrations increased significantly with dose ($p<0.001$, $r_s=0.956$) and mean concentrations were significantly different between all dose groups ($p<0.001$ for all comparisons). Brain THg concentrations for all dose groups are presented in Table 7.1.

7.3.2 Neurochemistry

Overall, levels of the NMDA receptor averaged 458.3 fmol/mg and ranged from 211.3 to 1002.0 fmol/mg, and levels of the $GABA_A$ (benzodiazepine) receptor averaged 164.6 fmol/mg and ranged from 44.1 to 385.5 fmol/mg. Activity of GS averaged 2983.8 (nmol/mg/min) and ranged from 2063.7 to 4184.3 (nmol/mg/min) and activity of GAD averaged 1270.0 (pmol/mg/h) and ranged from 217.0 to 2828.0 (pmol/mg/h). Biomarker values for males and females are presented in Table 7.2. Receptor levels and enzyme activity did not differ

significantly between males and females for any biomarker. Neurochemical biomarkers were also not significantly associated with animal age.

Brain THg concentrations were not associated with GS or GAD activity or levels of the NMDA or GABA receptor (Fig. 7.1) in a correlation or multiple regression after adjusting for age or gender. Enzyme activity and receptor levels did also not differ significantly between dose groups.

7.4 Discussion

Terrestrial songbirds are known to accumulate Hg, but potential neurological effects of this exposure had not previously been studied. In this study, we assessed four neurochemical biomarkers in juvenile zebra finches that were exposed to MeHg throughout development via maternal deposition and posthatch via contaminated food. We found that brain THg levels in the brains of juvenile offspring of females fed 0, 0.3, 0.6, and 1.2 μg/g MeHg spanned a wide range and reached up to 7.49 μg/g w.w. (equivalent to approximately 37.5 μg/g d.w.). These levels in finches are much higher than those found in embryos and hatchlings of other species following MeHgCl egg injection (Chapter 4, 5, 6,7), and are comparable to those linked to brain lesions in mallards (Heinz et al. 1976, Pass et al. 1975) and neurochemical changes in adult bald eagles and common loons (Rutkiewicz et al. 2011, Scheuhammer et al. 2008). Surprisingly, we did not find any relationship between the high levels of THg in the brain and the NMDA and $GABA_A$ (benzodiazepine) receptors or the GS and GAD enzymes even though developing embryos are generally considered to be very sensitive to toxicants (Grandjean et al. 2006). These findings demonstrate that

juvenile songbirds exposed *in ovo* and posthatch to MeHg may not exhibit subtle neurological effects.

In addition to providing information about the potential impacts of MeHg exposure on young songbirds, this work also supplements our previous egg injection studies (Chapter 5, 6) by evaluating neurochemical responses in a complementary avian model, the zebra finch, after developmental exposure via natural maternal deposition and diet in chicks that have reached the age of independence. This study design differs considerably from that of our egg injection studies, and the results may help to clarify some uncertainties that were raised with the egg injection work. Our previous studies involved injection of MeHgCl into fertile eggs followed by analysis of neurochemistry in embryos and young hatchlings. In our egg injection studies, we were unable to reproduce the Hg associated changes in the NMDA receptor seen in older bald eagles and common loons. Because our studies were limited to white leghorn chicken embryos and hatchlings, Japanese quail embryos, and thick-billed murre embryos, we questioned whether responses might differ by species. Here we studied a new species and again found that we did not reproduce the neurochemical changes found in wild birds. Our previous laboratory work investigated neurochemical changes in embryos and hatchlings with only developmental exposure. This study focused on slightly older birds, juveniles approaching the age of independence, and demonstrated that birds of this age also do not appear to experience neurochemical alterations as a result of MeHg exposure. Although the zebra finches were several weeks older than the embryos and hatchlings, we still cannot rule out the possibility

that our lack of neurological effects is due to the long latency period that is common with MeHg, as effects in humans can often take years to become apparent (Rice 1996, Spyker 1975). One important difference between the current study and our previous studies is the type of exposure to which the birds were exposed. In all studies, birds were exposed developmentally, but egg injection studies involved the artificial introduction of MeHg into fertilized eggs, while the zebra finch study involved natural deposition of MeHg, most likely bound to incorporated into proteins in the albumen (Nishimura et ala. 1976), into the egg as it was formed. The zebra finches were also maintained on a MeHg treated diet until tissue sampling. Even with this more realistic exposure, we did not identify Hg associated neurochemical changes therefore suggesting that the lack of effects in the egg injection studies may not be attributed simply to the unrealistic exposure.

In summary, we found that zebra finches exposed to maternally deposited MeHg accumulated high levels of brain THg, but did not display associations with four neurochemical biomarkers. These findings indicate that juvenile songbirds may not suffer neurological damage resulting from developmental and early life exposure. Together with our previous egg injection studies, these findings also reinforce that long term, rather than *in ovo* and early life exposure, may have the greatest impact on neurological health in birds. However, in this study we did not examine brains from adult birds to determine if they display neurochemical alterations. Considering that many adult terrestrial songbirds have elevated Hg exposures and related health effects (Hallinger et al. 2010, Hallinger et al. 2011,

Jackson et al. 2011, Janssens et al. 2003) this is an area that warrants future research.

Figures and Tables

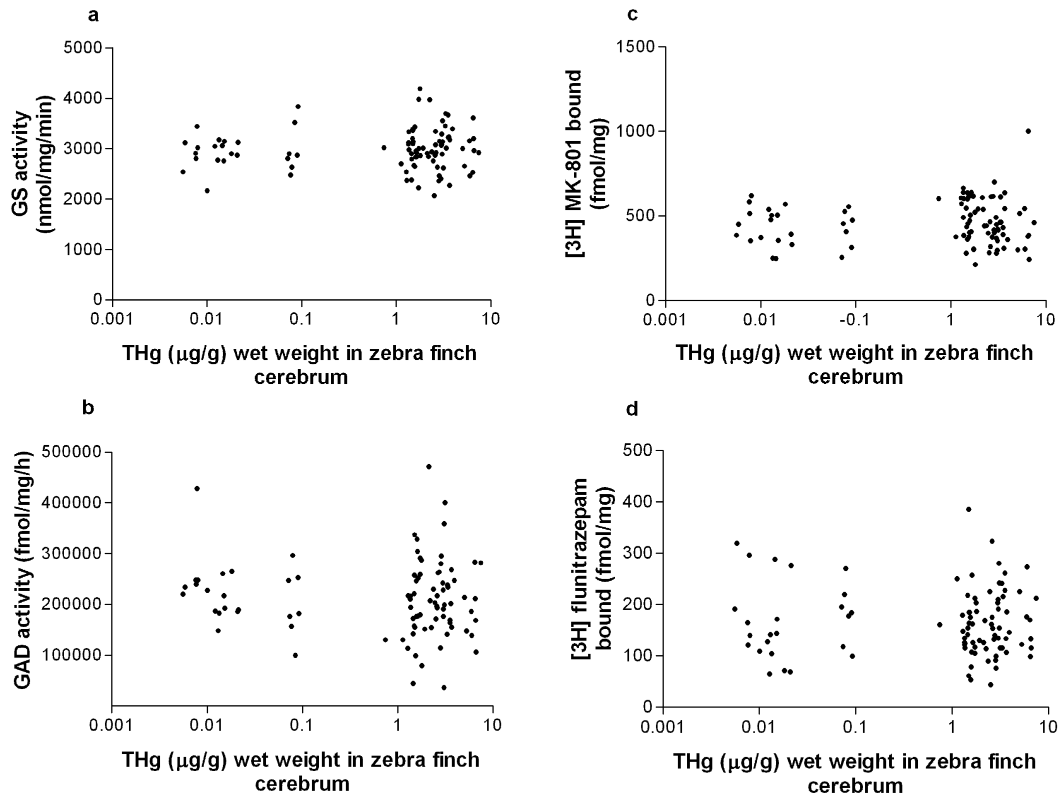

Figure 7.1 Brain mercury and neurochemistry in 50 day old zebra finches exposed to methylmercury cysteine by maternal deposition. Associations between total mercury (THg) in the cerebrum and **a** glutamine synthetase (GS) activity, (r_s=0.128, p=0.225, n=92), **b** glutamic acid decarboxylase (GAD) activity(r_s=-0.039, p=0.711, n=92), **c** N-methyl-D-aspartic acid (NMDA) receptor levels (r_s=-0.088, p=0.297, n=95), and **d** γ-aminobutyric acid ($GABA_A$) receptor levels (r_s=0.036, p=0.731, n=95) in whole brain of 44-56 day old offspring of female zebra finches fed 0, 0.3, 0.6, and 1.2 µg/g methylmercury cysteine

Table 7.1 Mercury in zebra finch brain. Mean total mercury concentrations (mean ±SD) in whole brain of approximately 50 day old zebra finches hatched from eggs of females that were fed a diet treated methylmercury cysteine Concentrations differed significantly between all dose groups

Dose (μg/g)	**Mean (μg/g)**	**Range (μg/g)**	**n**
0	0.06±0.15	0.006-0.75	25
0.3	1.53±0.18	1.13-1.93	29
0.6	2.99±0.47	2.14-3.95	33
1.2	6.22±0.74	5.06-7.49	9

Table 7.2 Neurochemical receptor and enzyme values in zebra finches. Values (mean ± SD) for glutamine synthetase (GS) activity, glutamic acid decarboxylase (GAD) activity, N-methyl-D-aspartic acid (NMDA) receptor levels, and γ-aminobutyric acid ($GABA_A$) receptor levels in whole brain of approximately 50 day old male and female offspring of female zebra finches fed 0, 0.3, 0.6, 1.2 µg/g methylmercury chloride. Sample sizes are in parenthesis

	GS (nmol/mg/min)	GAD (pmol/mg/h)	NMDA (fmol/mg)	GABA (fmol/mg)
Overall	2983.8 ± 402.9 (92)	211.7 ± 73.5 (92)	458.3 ± 131.2 (95)	164.6 ± 66.2 (95)
Male	3035.0 ± 451.4 (46)	210.3 ± 58.9 (46)	471.7 ± 131.9 (48)	158.8 ± 61.1 (48)
Female	2932.6 ± 345.1 (46)	213.2 ± 86.4 (46)	444.6 ± 130.4 (47)	170.4 ± 71.2 (47)

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Chapter 8

Postmortem stability of brain GABAergic and glutamatergic receptors and enzymes under ecological conditions[3]

8.1 Introduction

Many environmental contaminants are potent neurotoxicants that have the potential to cause adverse effects. In recent years, biochemical alterations have been applied as neurochemical biomarkers in ecological studies to assess the neurological impacts of environmental contaminants in fish and wildlife populations (Basu et al., 2012). These studies involve several different species and characterize responses to a variety of toxicants, including metals (Basu et al., 2009; Rutkiewicz et al., 2011) wastewater effluents (Gagne et al., 2007) and biological toxins (Nam et al., 2010). In some cases, such as regular herring gull monitoring studies (Rutkiewicz et al., 2010) or sanctioned polar bear hunts (Basu et al., 2009), researchers may carefully design the study so that brain tissues may be extracted from freshly harvested animals and properly stored frozen until biomarker analysis. However, in a majority of cases such is not possible as tissues are extracted from field-collected carcasses that have experienced varying postmortem conditions. In such studies, individuals within the same study may be exposed to a variety of different environmental and storage conditions that may affect cellular integrity and

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biomarker values. Accordingly, in ecotoxicology studies biomarkers including receptor binding and enzyme activity in wildlife tissues may vary depending length of time and ambient temperature in the field after an animal's death, length and temperature of storage, and the number of freeze thaw cycles the samples have undergone. Therefore it is important to understand potential time and temperature dependent changes in neurochemical biomarkers when designing and interpreting results from field-based studies.

The postmortem stability of neurochemical biomarkers has received some attention. Most notably, several biomedical studies have used rodent models to evaluate the postmortem stability of neurochemical receptors and enzymes (Bhargava et al., 1986; Syapin et al., 1987; Whitehouse et al., 1984) that are often studied in human autopsy tissues. These studies find that certain receptors and enzymes maintain their integrity after death and storage; however, the relevance of these studies to ecotoxicology are not clear as they typically mimic hospital conditions and may not accurately reflect the tremendously variable and unpredictable natural environment in which carcasses are obtained.

Recently, ecological studies have started to address the stability of neurochemical biomarkers. Fish brain acetylcholinesterase activity is quite stable across a range of postmortem conditions (Phillips et al., 2002). Using mink brain, Stamler et al. (2005) studied the effects of relevant storage conditions on a number of cholinergic and dopaminergic biomarkers. Components of GABA and glutamate neurotransmission have yet to be evaluated in terms of their postmortem stability, despite the fact that they are, respectively, the primary inhibitory and excitatory

neurotransmitters across vertebrates, and that they regulate ecologically important behaviors like learning , memory (Reis et al., 2009), and reproduction (Waye and Trudeau, 2011). Further, a number of recent studies have shown that changes in brain GABAergic and glutamatergic receptors and enzymes are associated with toxicant exposure (Basu et al., 2009; Gagne et al., 2007; Rutkiewicz et al., 2011; Scheuhammer et al., 2008).

The current study uses an approach similar to that of Stamler et al. (2005) to investigate, using the embryonic chick brain as a model, the stability of receptors and enzymes involved in GABA and glutamate neurotransmission under various storage and postmortem environmental conditions. We have chosen the chick as a model since it is a common laboratory model in avian toxicology, and as a bird it complements our on-going work concerning the effects of environmental neurotoxicants on wild birds. Given sequence and structural similarities of neurochemicals among vertebrates (Bosma et al., 1999; Kurosawa et al., 1994) our findings may also apply to other species. The overall goal of this work is to evaluate the stability of the *N*-methyl-D-aspartate (NMDA) and γ-aminobutyric acid ($GABA_A$) (benzodiazepine) receptors and glutamine synthetase (GS) and glutamic acid decarboxylase (GAD) enzymes to determine if they can be measured in tissues exposed to variable conditions, how to appropriately store tissues for such analyses, and how to interpret data from samples exposed to differing environmental and field conditions. This work affords field investigators a better understanding of the suitability and limitations of these neurochemical biomarkers in ecological studies.

8.2 Methods

8.2.1 Tissues

White leghorn chicken embryos were selected as a model for wild birds because they are a readily available source of fresh avian tissue, their size is convenient for performing various postmortem exposures in the laboratory, and they are routinely used in avian toxicology. Embryos were acquired as fresh fertilized eggs (Michigan State University Poultry Teaching and Research Center) that were artificially incubated (37.7 °C, 50% humidity) in the laboratory. At day 19, embryos were decapitated and heads were subject to experimental postmortem conditions. Immediately following exposure to varying postmortem conditions, whole brains were removed and processed for neurochemical assays. Baseline samples were also prepared with fresh whole brains from embryos that were decapitated and immediately processed.

8.2.2 Postmortem Conditions

To test the effects of postmortem time and temperature, embryonic heads were placed at 7 °C in a standard Danby refrigerator or 25 °C in a Fisher Scientific Isotemp incubator for 12, 24, or 48 h. These conditions were selected to represent temperatures typical during spring and summer field collection seasons of temperate ecosystems, and time periods during which carcasses may be collected before becoming decayed. To test the effects of storage time and temperature, heads were stored in a Kelvinator -20 °C freezer (non-frost free) or a Revco -80 °C freezer for 7, 28, or 56 days. These are storage temperatures commonly available to

scientists, and the times were selected to represent both short and long-term storage. Stored carcasses are often thawed in order to subsample tissues. To test the effects of freeze thaw cycles, heads were subject to either one or two freeze thaw cycles. Heads were frozen at -20 °C for 24 hours, thawed at room temperature, then processed or the procedure was repeated for a second freeze thaw cycle. For each condition a sample size of 6-8 embryos was used. As previously mentioned, baseline samples were included in each study.

8.2.3 Neurochemical Assays

Cellular homogenates, enzyme supernatants, and membranes were prepared in whole brain using previously described methods (Rutkiewicz et al., 2011). Prepared samples were stored at -80 °C until analyses. GAD activity was assayed in brain homogenate and GS activity was assayed in enzyme supernatant according to previously described protocols (Rutkiewicz et al., 2011). For enzyme assays, samples were assayed in duplicate and a pooled control sample was used to monitor variability, as measured by percent relative standard deviation (%RSD), between replicates and between sets of assays. For the GS assay, variability between replicates averaged 4% and between set variability was 8%. For the GAD assay, variability between replicates averaged 8% and between set variability was 15%. Neurochemical receptors were assayed in cellular membranes, using [3H] MK-801 (NMDA receptor) and [3H] flunitrazepam ($GABA_A$ benzodiazepine receptor) for radioligands, as previously described (Rutkiewicz et al., 2011). Samples for binding assays were assayed in quadruplicate and variability was monitored using a pooled

control sample. Between plate variability averaged 15% for the NMDA receptor and 20% for the GABA receptor

8.2.4 Statistical Analysis

All analyses were performed using PASW Statistics (V.17.0, Chicago, IL, USA). One way ANOVA, followed by Tukey's post hoc test when appropriate, was used to compare enzyme activity and receptor binding between treatments. A P-value of <0.05 was considered significant.

8.3 Results

8.3.1 Effects of Postmortem Temperature and Time

At both 7 °C and 25 °C, [3H] MK-801 binding to the NMDA receptor remained stable over 48 hours (Fig. 8.1a). [3H] flunitrazepam binding to the $GABA_A$ benzodiazepine receptor decreased steadily with time when stored at 7 °C, and binding varied significantly between baseline and 48 hours (Fig. 8.1b). After 12 hours at 25 °C, binding decreased nonsignificantly to approximately 60% of baseline, where it remained through 48 hours. Binding varied between the two postmortem temperatures at 48 hours. GS activity increased significantly relative to baseline at both temperatures in the first 12 hours, then decreased to levels approximately 75% of baseline (Fig. 8.1c). GAD activity also increased initially then decreased to approximately 75% of baseline by 24 hours at 7 °C (Fig. 8.1d). At 25

°C, activity was reduced significantly at 24 hours but was otherwise stable. Activity at 24 hours differed significantly between samples kept at 7 °C and 25 °C.

8.3.2 Effects of Storage Temperature and Time

[3H] MK-801 binding to the NMDA receptor did not vary significantly in samples stored at -20 °C or -80 °C for up to 56 days (Fig. 8.2a). On the contrary, binding of [3H] flunitrazepam to the $GABA_A$ benzodiazepine receptor decreased significantly after 7 days of storage at -80 °C, and continued to decrease nonsignificantly to approximately 40% of baseline at 28 and 56 days (Fig. 8.2b). Binding also decreased with storage at -20 °C, and significant differences were found between baseline and samples stored for 28 days, although samples stored for 7, 28, and 56 days did not differ significantly. GS activity in samples stored at -80 °C decreased to 70% of baseline after 7 and 28 days, and continued to decrease significantly to less than 40% of baseline after 56 days (Fig. 8.2c). Activity decreased similarly with storage at -20 °C and significant differences were found between timepoints. GAD activity was fairly stable at -80 °C. Though activity decreased gradually to 80% of baseline by 56 days this was not to a level of statistical significance (Fig. 8.2d). In samples held at -20 °C, activity decreased significantly to 40% of baseline by 56 days, although activity did not differ significantly between 7, 28, or 56 day timepoints. GAD activity differed significantly between samples stored at -20 °C and those stored at -80 °C at all timepoints.

8.3.3 Effects of Freeze Thaw Cycles

[3H] MK-801 binding to the NMDA receptor was not affected by either one or two freeze thaw cycles (Fig. 8.3a). [3H] flunitrazepam binding to the $GABA_A$ benzodiazepine receptor decreased significantly to less than 20% of baseline with one or two freeze thaw cycles (Fig. 8.3b). GS activity decreased significantly to approximately 50% of baseline after one or two freeze thaw cycles (Fig. 8.3c). GAD activity was reduced to approximately 80% of baseline with either one or two freeze thaw cycles, but this decrease was not to a level of statistical significance (Fig. 8.3d). Neither receptor binding nor enzyme activity differed between samples that underwent one or two freeze thaw cycles.

8.4 Discussion

Receptors and enzymes involved in glutamate and GABA neurotransmission represent attractive neurochemical biomarkers in wildlife due to their roles in ecologically important behaviors. By investigating contaminant-associated changes in these biomarkers in wildlife, researchers may identify neurological damage before it has progressed to such behavioral changes. This study is the first to evaluate the stability of four components of these pathways in birds under relevant environmental conditions. This work demonstrates that the avian NMDA receptor, GABA receptor, GS, and GAD may be successfully measured in tissues held under suboptimal conditions, but also highlights the differences in stability of the four biomarkers and emphasizes the importance of consideration of environmental factors when interpreting data from field based studies. In general, [3H] MK-801

binding to the NMDA receptor was stable across multiple postmortem conditions, whereas binding [3H] flunitrazepam binding to the $GABA_A$ benzodiazepine receptor and activity of GS and GAD displayed time and temperature dependent alterations.

Postmortem environmental conditions, such as the time and temperature between death and sampling, are often unknown in studies of wildlife but may greatly impact receptor and enzyme function. In this study, we investigated receptor binding and enzyme activity in samples exposed to typical temperate spring and summer temperatures for up to 48 hours. We found that [3H] MK-801 binding to the NMDA receptor was extremely stable under these conditions. Similar to our results, studies of NMDA receptor binding in human autopsy tissue have found that binding is not affected by postmortem delays of up to 36-74 hours (Sundstrom et al., 1997; Piggott et al., 1992; Quarum et al., 1990). Surprisingly, [3H] flunitrazepam binding to the $GABA_A$ (benzodiazepine) receptor decreased steadily at 7 °C, but decreased nonsignificantly then remained relatively stable at 25 °C. This variability is similar to rodent studies that demonstrate that in some cases binding to the GABA receptor remains constant over time (Atack et al., 2007), while in other cases it remains constant at cooler temperatures but increases at room temperature (Syapin et al., 1987; Whitehouse et al., 1984). Despite the significant changes that we did observe, binding of samples exposed for 12 and 24 hours did not differ from baseline samples at either temperature, and binding at 7 °C did not differ from binding at 25 °C at these two timepoints. These findings suggest that $GABA_A$ (benzodiazepine) binding data may be comparable between samples collected within 24 hours. Unlike human and rodent studies demonstrating that GS

is not significantly affected by various postmortem conditions (Burbaeva et al., 2003; Vogel et al., 1975; Bhargava et al., 1986; Ritchie et al., 1986), we found that GS activity was altered and increased in variability at both temperatures. The numerous significant differences, particularly in samples held at 25 °C, suggest that GS may not be an ideal biomarker in wildlife tissues collected under warmer weather conditions. Many studies in rodents have demonstrated that GAD is fairly stable under various postmortem and conditions (Fahn et al., 1976; Bhargava et al., 1986; Vogel et al., 1975), but our findings more closely align with those of Puymirat et al. (1979), who did observe modest declines, especially in samples exposed to warmer postmortem and storage temperatures. Because few significant differences in activity were found between timepoints, GAD may be considered fairly stable under many relevant environmental conditions, although differences between samples held at the two temperatures for 48 hours suggests that carcasses sampled after prolonged periods in the field during different seasons may not be comparable.

Even under ideal freezer storage, receptors and enzymes may degrade over time. This may create difficulties in measuring receptor levels and enzyme activity, and complicate data analysis for samples with unknown or differing storage histories. Previously, several components of the cholinergic and dopaminergic pathways were found to be stable for up to four weeks under various storage conditions (Stamler et al., 2005). In this study, we found that [3H] MK-801 binding to the NMDA receptor in the chicken was stable at both -80 °C and -20 °C for up to eight weeks, and based on a human study demonstrating NMDA receptor stability for years (Piggott et al., 1992), may be stable for extended storage periods. [3H]

flunitrazepam binding to the $GABA_A$ (benzodiazepine) receptor in rodents was not significantly affected by short-term storage up to 72 hours (Syapin et al., 1987). In the current study, this receptor was less stable relative to baseline in stored embryonic chicken brains, but the fact that levels did not vary significantly between samples stored for one, four, or eight weeks suggests that much of the damage is due to the initial freeze thaw and that samples held for longer periods may yield comparable results. Binding was similar between samples stored at each temperature, but was more variable at -20 °C, which suggests colder storage is ideal. Although human and rodent studies have found that GS is stable in autopsied tissues for up to 40 months at -20 °C (Carter et al., 1982) or in experimental rat tissues for 24 hours at -80 °C (Vogel et al., 1975), we found that GS activity decreased significantly by eight weeks at both temperatures and might be best studied in fresh samples. Because variability increased in samples stored at -20 °C, if short term storage is required it should be done at -80 °C. Similar to what was seen in rodents (Puymirat et al., 1979), GAD activity was stable at -80 °C and significantly less stable at -20 °C. Activity did not fluctuate greatly within temperature groups, which indicates that samples with identical storage temperatures may be comparable. However, variability did increase somewhat in samples stored at -20 °C. Our findings and those of Stamler et al. (2005) suggest that long-term storage at -80 °C is ideal for tissues intended for most neurochemical biomarker studies, as some biomarkers are less stable with warmer storage, but that -20 °C is acceptable for

many biomarkers and this may be a more economical and feasible option in field situations.

Because field-collected samples are typically frozen between collection and processing, they are susceptible to degradation upon thawing. Additionally, samples may undergo further freeze thaw cycles when being sub-sampled for various studies or transferred between facilities. As with other postmortem conditions tested, stability varied between biomarkers. The binding of [3H] MK-801 to the NMDA receptor again demonstrated stability, with little change after one or two freeze thaw cycles, as did GAD, with only a minimal decrease in activity after one or two freeze thaw cycles. Both $GABA_A$ receptor binding and GS activity decreased significantly following one freeze thaw cycle, but did not differ between one or two cycles. These findings are similar to those of Stamler et al. (2005), who likewise found little difference in receptors and enzymes beyond the first freeze thaw cycle. These findings suggest that this degradation should not impact analysis in most studies, provided that all samples have been previously frozen.

In summary, this study found that components of the GABA and glutamate neurotransmission pathways can likely be measured successfully in studies of field-collected samples, but effects of postmortem environmental and storage conditions did vary and may impose important limitations on their use in such studies. Of the four biomarkers investigated in this study, [3H] MK-801 binding to the NMDA receptor was remarkably stable under all conditions and can likely be studied in field-collected samples with little regard for postmortem or storage conditions. This is particularly important given that several recent studies have documented

toxicant-associated changes in brain NMDA receptor levels (Basu et al., 2009; Rutkiewicz et al., 2011; Scheuhammer et al., 2008). [3H] flunitrazepam binding to the $GABA_A$ (benzodiazepine) receptor displayed some changes with environmental and storage conditions but few were significant, though it appears that only field samples collected 12-24 hours after the animal's death may be reliable for studies using this receptor as a biomarker. As the carcasses most often used in studies must be collected shortly after death, before the tissues are visibly decayed, this restriction may not greatly limit wildlife studies. However, it is important to note that variability of this receptor was fairly high under all conditions and in some cases may exceed toxicant induced changes, therefore masking subtle relationships. Of the biomarkers studied here, GS may be least appropriate for study in wildlife tissues despite its stability in biomedical studies (Carter et al., 1982; Vogel et al., 1975; Burbaeva et al., 2003; Bhargava et al., 1986; Ritchie et al., 1986). Both postmortem environmental and storage conditions significantly altered activity, suggesting that this enzyme might best be suited as a biomarker in studies of animals with well known and comparable postmortem histories. The enzyme was altered significantly when held at 25 °C but not at 7 °C, which indicates that it may not be appropriate for study in samples collected during warmer months. Because GAD was significantly affected by storage temperature, studies involving this enzyme may be limited to samples known to have similar storage histories.

Although we addressed several factors concerning the effects of postmortem environmental and storage conditions on neurochemical receptors and enzymes, one must also bear in mind that other factors must be considered when applying

neurochemical biomarkers in wildlife toxicology studies. For example, neurochemistry can vary due to age (Kito et al., 1990), gender (Frick et al., 2002), and perhaps unstudied factors like breeding status or season. Ideally in studies of wildlife, analysis should be controlled for or animals should be matched for such factors (e.g., through selective sample selection or gender/age identification through necropsy, plumage etc.) whenever possible.

In light of our findings here, it is important to exercise caution when comparing samples with different histories because combinations of environmental and storage factors may magnify nonsignificant differences or mask significant changes. High levels of variability for some biomarkers may also present challenges. We tested only a common set of environmental conditions in a temperate ecosystem in our study, but as postmortem conditions may be completely unknown in ecological studies it is possible that some wildlife samples are exposed to conditions even more extreme than those we tested. Though we did find that some receptors and enzymes are altered by postmortem conditions, an important factor to consider is that our study involved chicken embryos, which have a thin skull that may not offer the brain the same protection of the thick fully calcified skull of a wild bird. There may also be inter-species differences between birds in neurochemical stability that were not addressed here. Though sequences and structures of neurochemical receptors and enzymes are often similar among vertebrates, anatomical differences between birds and other animals may also influence postmortem stability and may preclude the application of our findings to wild mammals or fish. Nevertheless, this work demonstrates stability of some

biomarkers and provides insight into factors that must be considered with less stable biomarkers, and supports the potential use of some neurochemical biomarkers for predicting neurological damage in wildlife.

Figures

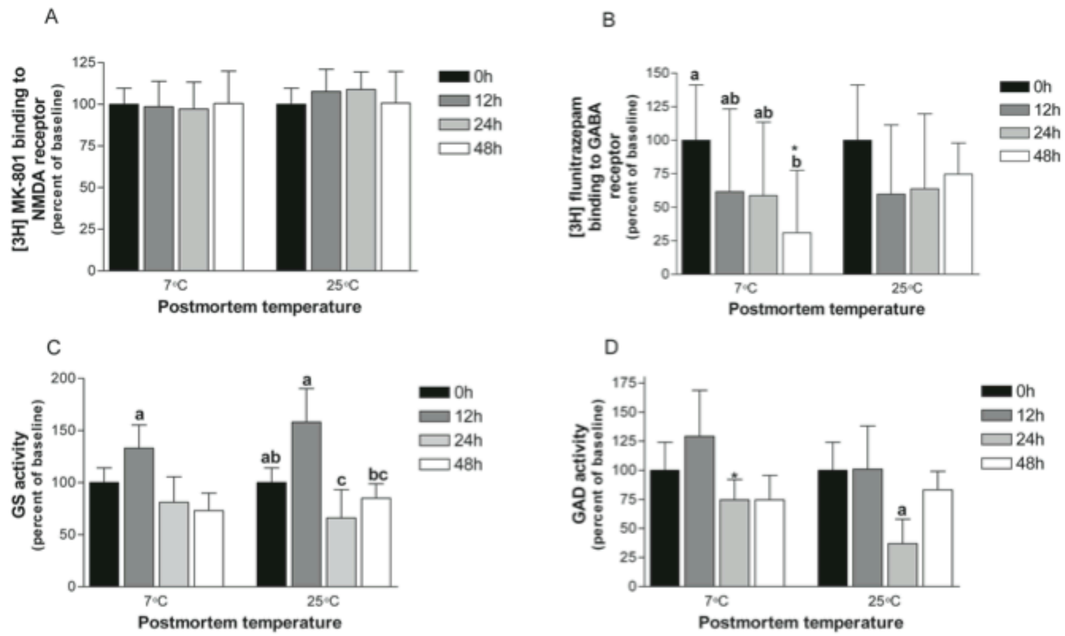

Figure 8.1 Effects of postmortem environmental conditions on neurochemical biomarkers in chicken brain. Mean (± standard deviation) levels of the *N*-methyl-D-aspartic acid (NMDA) receptor (A) and γ-aminobutyric acid ($GABA_A$) receptor (B), and activity of glutamine synthetase (GS) (C) and glutamic acid decarboxylase (GAD) (D), relative to baseline, in embryonic chick brain held at 7 °C or 25 °C for 12, 24, or 48 hours. Letters represent significant differences, according to Tukey's post hoc test, in receptor levels or enzyme activity between sampling timepoints within a temperature category. Bars that do not share a common letter are significantly different. Asterisks indicate that receptor levels differed significantly between temperature categories for that sampling timepoint. Sample sizes for each bar are 7-8 embryos

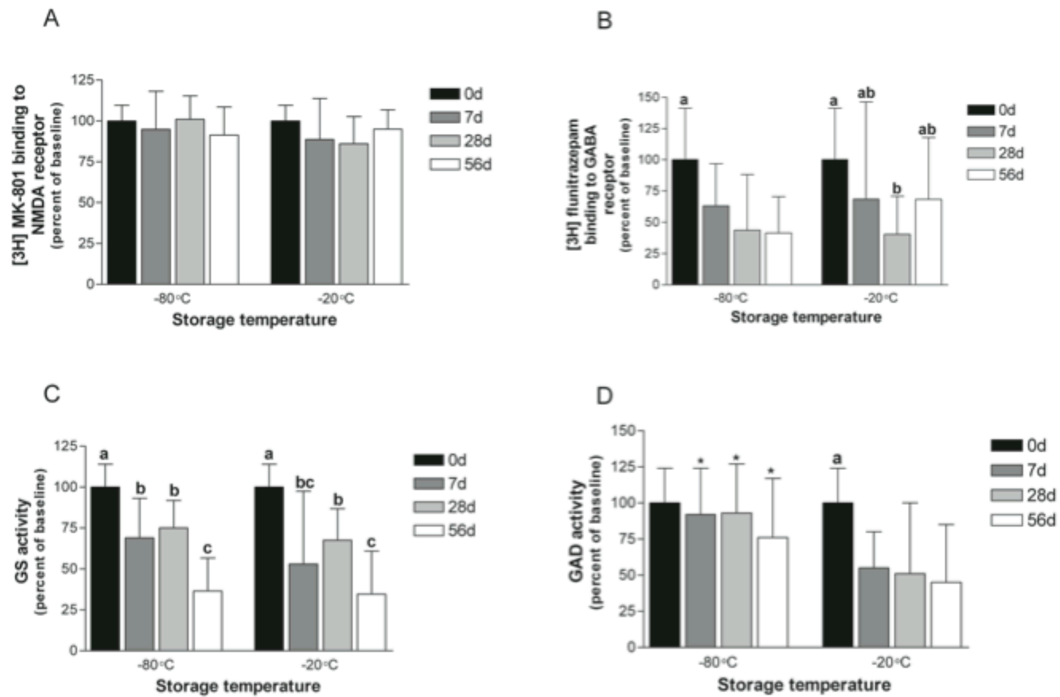

Figure 8.2 Effects of carcass storage conditions on neurochemical biomarkers in chicken brain. Mean (± standard deviation) levels of the *N*-methyl-D-aspartic acid (NMDA) receptor (A) and γ-aminobutyric acid ($GABA_A$) receptor (B), and activity of glutamine synthetase (GS) (C) and glutamic acid decarboxylase (GAD) (D), relative to baseline, in embryonic chick brain stored at -80 °C or -20 °C for 7, 28, or 56 days. Letters represent significant differences, according to Tukey's post hoc test, in receptor levels or enzyme activity between sampling timepoints within a temperature category. Bars that do not share a common letter are significantly different. Asterisks indicate that receptor levels differed significantly between temperature categories for that sampling timepoint.. Sample sizes for each bar are 6-8 embryos

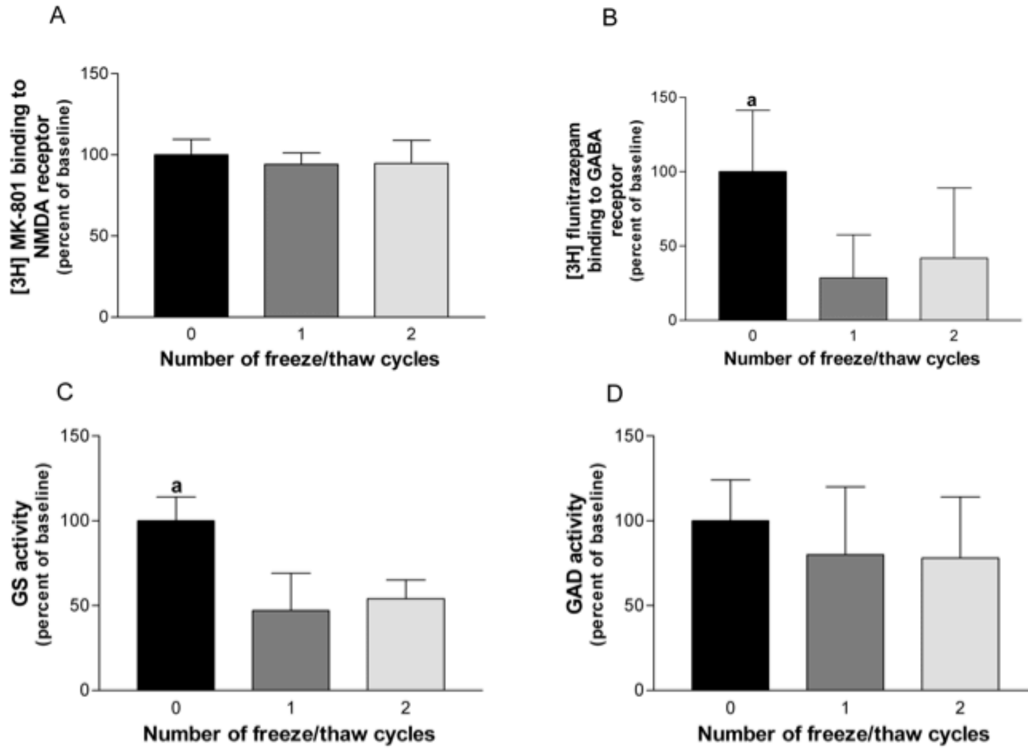

Figure 8.3 Effects of freeze thaw cycles on neurochemical biomarkers in chicken brain. Mean (± standard deviation) levels of the *N*-methyl-D-aspartic acid (NMDA) receptor (A) and γ-aminobutyric acid ($GABA_A$) receptor (B), and activity of glutamine synthetase (GS) (C) and glutamic acid decarboxylase (GAD) (D), relative to baseline, in embryonic chick brain after one or two freeze thaw cycles. Letters represent significant differences, according to Tukey's post hoc test, in receptor levels or enzyme activity between samples with 0, 1, or 2 freeze thaw cycles. Bars that do not share a common letter are significantly different. Sample size is 8 embryos for each bar

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Chapter 9

Conclusion

9.1 Objective and Significance

In my dissertation work I sought to evaluate and apply neurochemical receptors and enzymes involved in glutamate and γ-aminobutyric acid (GABA) neurotransmission as biomarkers for assessing mercury (Hg) risks in birds. The overall hypothesis was that exposure to environmentally relevant levels of Hg induces subclinical changes in receptors and enzymes involved in these pathways, and that these changes may warn of clinical and behavioral impacts (Fig. 9.1). The project applied a comprehensive approach by exploring for Hg associated neurochemical changes in wild juvenile adult and birds with long term dietary Hg exposure, as well as for Hg associated neurochemical and behavioral changes in laboratory embryos and hatchlings developmentally exposed to Hg under controlled settings. This project also evaluated the stability of neurochemical biomarkers under ecologically relevant conditions to determine their suitability for use in field based studies. Such information about the neurological impacts of Hg is important given that Hg exposure is of growing concern for avian populations. Large numbers of several avian species have been found to have elevated tissue Hg concentrations or reside in regions with high levels of Hg deposition (reviewed in Scheuhammer et al. 2007, USEPA 1997). Mercury levels are increasing in many parts of the globe

(Sprovieri et al. 2010), and groups worldwide, including the U.S. EPA, are currently reexamining legislation regarding Hg. The science based evidence presented in this dissertation will help to aid policy makers who are now reevaluating this chemical.

In terms of research on wild birds, the work presented here increases our knowledge of Hg associated neurochemical changes with environmental exposure by studying neurochemical biomarkers in two species (the herring gull, which had not previously been studied with regards to Hg toxicity, and the bald eagle) from across the US Great Lakes, and several new and previously studied biomarkers (the nicotinic acetyleholine (nACh), muscarinic acetylcholine (mACh), *N*-methyl-D-aspartate (NMDA), and GABA receptors, and glutamic acid decarboxylase (GAD) and glutamine synthetase (GS) enzymes).

In terms of research on laboratory birds, the studies here are among the first to evaluate the neurological impacts of *in ovo* methylmercury (MeHg) exposure in birds, and examine the effects of MeHg on multiple species (white leghorn chicken, Japanese quail, thick-billed murre, and zebra finch), in both whole brain and discrete brain regions (cerebrum, cerebellum, and optic lobe), and at several developmental timepoints (embryonic days 10, 14, and 19, and posthatch days 1 and 7). In the various studies an integrative approach was taken whereby several tiers of neurological outcomes (neurochemistry, neuropathology, and neurobehavior) are explored. Additionally, postmortem and storage conditions that may impact biomarkers and limit their utility in field collected tissues are discussed.

The significance of this work is that it helps to establish thresholds for Hg associated neurochemical changes in wild birds and provides information about the

potential for neurological effects with developmental exposure. It also helps to increase our understanding of toxicant responses and neurochemical differences across a range of avian species and exposure scenarios and during several timepoints during embryonic and posthatch development.

9.2 Major Results and Discussion

9.2.1 Mercury Exposure and Neurochemistry in Juvenile and Adult Wild Birds

9.2.1.1 Exposure

In this dissertation, Hg exposure and relationships with neurochemical biomarkers were explored in two wild avian species collected from across the US Great Lakes. The herring gull and the bald eagle have both been studied fairly extensively with regards to Hg exposure, but brain Hg in the herring gull has not been studied previously and only one study has investigated brain Hg and potential neurological impacts bald eagles from across Canada (Scheuhammer et al. 2008). Herring gulls and eagles are long lived piscivorous birds likely to accumulate Hg through their diets, and both are year round residents in the Great Lakes (Fox et al. 2001), thus their tissue Hg concentrations are likely to be indicative of contamination in the region. While the bald eagle is a top predator, the herring gull is considered to be a more opportunistic piscivore that supplements with terrestrial foods (Fox et al. 2001). Therefore the studies presented in this dissertation provide a valuable assessment of two Great Lakes species with slighty different diets. Not surprisingly, considering that fish are the main source of Hg exposure in wild birds, brain Hg levels in herring gulls were found to be quite low, with a mean

concentration of 0.5 μg/g dry weight (d.w.) and a maximum of only 2.0 μg/g. On the other hand, brain Hg levels in bald eagles averaged 2.8 μg/g with a maximum of 34 μg/g. Liver Hg concentrations were also quite high in bald eagles. On average, livers contained 8.0 μg/g Hg and 17% of birds exceeded a conservative toxic threshold of 16.7 μg/g. (Zillioux et al. 1993). In the eagle study, correlation analysis revealed that liver and breast feather total Hg (THg) were fairly well correlated with brain THg hence they may be used to predict brain THg in studies where only liver, which is commonly sampled for Hg exposure, or breast feathers, which can be sampled nonlethally, values are available. Both studies demonstrated geographic variation, with concentrations in herring gulls highest at the easternmost site perhaps due to atmospheric transport. Concentrations in eagles varied between states and were highest in birds from Michigan, which may result from any number of factors including diet, proximity of emissions sources, or geochemical factors. Overall, these two studies indicate that some bald eagles, but not herring gulls, across the Great Lakes may be at risk for Hg related effects (Fig. 9.2).

9.2.1.2 Neurochemistry

The vastly different ranges of brain Hg in herring gulls presented the opportunity to compare neurochemical relationships with Hg in two species with different levels of exposure. In the herring gull study, no relationship was found between brain Hg concentrations and levels of the NMDA or muscarinic or nicotinic cholinergic receptor despite the fact that the muscarinic receptor and NMDA

receptor have been associated with Hg in other avian species (Scheuhammer et al. 2008). In the bald eagle study, brain THg was negatively associated with the NMDA receptor and positively associated with GS activity, which is consistent with findings in birds (Scheuhammer et al. 2008) and rodents (Kung et al. 1989). Interestingly, a negative association between inorganic Hg (IHg) and GAD activity was found, and associations with the NMDA receptor and GS were significant for IHg but not OHg. Inorganic Hg is known to be a more potent toxicant than OHg in vitro (Basu et al. 2006, 2010), and the results of the eagle study may indicate that following demethylation in the brain, IHg is the primary toxicant. It may also reflect a latency period for toxicity, as birds with high levels of IHg have been exposed for long enough for demethylation to occur. Considering that glutamate and GABA have roles in behaviors that could be important for a birds ability to survive and reproduce (Gibbs et al. 2005, Scholes 1965), changes such as those identified in the NMDA receptor, GS, and GAD have potential implications at the population level.

9.2.1.3 Thresholds and Potential Impacts in Wild Birds

The two wildlife studies included in this dissertation, in conjunction with two studies recently published by colleagues (Hamilton et al. 2012, Scheuhammer et al. 2008), suggest that a threshold for neurochemical changes in wild exists. The identification of such thresholds for neurotoxicity is important for risk assessment because it allows investigators to predict, based on contaminant levels in individuals, the potential for detrimental effects. The four studies are summarized in Table 9.1 may begin to form the foundation for deriving such a threshold in wild

birds. This dissertation describes a study of herring gulls that displayed very low levels of Hg exposure relative to other wild avian species (Table 2.2) and no associations with neurochemical receptors, and a study of bald eagles with much higher levels of Hg and associations with the NMDA receptor, GS, and GAD. Studies performed by Environment Canada have found associations between brain Hg and the NMDA and muscarinic cholinergic receptor in bald eagles and common loons with Hg levels comparable to those in the bald eagle study from this dissertation, and no associations in common loons with much lower Hg levels (Table 9.1). Based on these studies, a threshold for neurochemical changes in wild birds likely lies between the relatively low THg concentrations seen in brains from herring gulls (maximum 2.0 μg/g d.w.) and some common loons (maximum 3.7 μg/g d.w.) and the higher concentrations seen in bald eagles (maximum 34 μg/g d.w.) and other common loon (maximum 68 μg/g d.w.). A threshold of approximately 5-10 μg/g d.w. for neurochemical changes in wild birds may be plausible considering these findings and other studies demonstrating that in wild mammals clinical signs of Hg toxicity are often apparent with brain Hg levels above 25 μg/g d.w. (converted from wet weight (w.w.) assuming 80% moisture), and neurochemical changes are apparent with lower levels (reviewed in Scheuhammer et al. 2007). However, it is important to consider that this is only a tentative threshold that was estimated using studies of only three species and a limited number of biomarkers, and both species and biomarkers likely vary in sensitivity. Nevertheless, the ranges of brain Hg levels in several avian species reach or exceed this proposed threshold (Table

2.2), suggesting that many birds across the United States and Canada may be at risk for Hg associated neurochemical changes.

9.2.2 Mercury Exposure and Neurological Impacts in Laboratory Embryos and Hatchling Birds

9.2.2.1 Exposure

The work in this dissertation is the first to describe Hg distribution in the avian embryo and to track accumulation in tissues throughout development and post hatch in order to determine susceptible tissues and sensitive time windows for toxicity. Following day 0 air cell egg injection of MeHg chloride (MeHgCl), distribution of Hg in chicken embryos and hatchlings was similar to that of an adult bird, with highest concentrations in the kidney and liver, and lower but appreciable concentrations in brain, heart, and muscle. Mercury concentrations increased most rapidly in the last days of incubation through hatch as yolk was absorbed, but depuration into rapidly growing feathers minimized increases in tissues in the week after hatch. This study demonstrates that tissues such as the kidney and brain may be of toxicological concern in avian embryos and young hatchlings, and that the period surrounding pipping and hatching may be the most susceptible time period for developing birds.

9.2.2.2 Neurochemistry and Neuropathology

The neurological impacts of developmental MeHg exposure were explored in the laboratory at multiple levels in this dissertation. Neurochemical biomarkers

were assessed to identify the earliest subclinical changes that might occur in the brain of embryos and young hatchlings. A histological analysis was also done to determine if MeHg exposure in embryos results in the gross pathological changes typical to Hg toxicity.

Subclinical impacts on the NMDA receptor, $GABA_A$ (benzodiazepine) receptor, GS, and GAD were extensively evaluated using several species and experimental designs. Several age categories, ranging from the approximate midpoint of embryonic development through pipping, to young hatchlings and independent juveniles were studied, as well as whole brain and individual brain regions (cerebrum, cerebellum, optic lobe). The studies in chickens and quail involved air day 0 MeHgCl air cell injections into commercially purchased eggs, which is an artificial exposure method but exposes the eggs at the start of embryonic development. The thick-billed murre study involves injection into field collected eggs at incubation day 5, a day at which exposure by injection is known to be more toxic (Heinz et al. 2006). Finally, the zebra finch study involves deposition by experimentally exposed mothers followed by dietary exposure in chicks. The neurochemical findings for the studies included in this dissertation are summarized in Table 9.2. Despite the fact that brain THg concentrations reached levels comparable to those in wild birds that displayed neurochemical alterations (Table 9.1, Table 9.2), very few associations between brain THg levels or dose group were observed in these studies (Fig. 9.2). No associations were seen in embryonic quails, juvenile zebra finches, hatchling chickens, and most embryonic chickens. A significant association was seen between brain THg and GAD in whole brain of day

19 chicken embryos, but the relationship was weak. Significant associations were also seen between brain THg and GAD activity and the NMDA receptor of thick billed murred pipping embryos. This may indicate species differences in responses, but the relationship with GAD was fairly weak and displayed great variability, and the relationship with the NMDA receptor was not reproduced by colleagues (Braune et al. 2012). Associations that differed from findings in both bald eagles and chicken embryos from eggs injected with MeHgCl were seen in whole brain of day 19 chicken embryos from eggs injected with MeHgCys. This could indicate that responses vary based on the form of Hg exposure, but these findings are limited by the small sample size of this pilot study (n=14). Overall, these studies indicate no consistent relationship between developmental MeHg exposure and these four neurochemical biomarkers. Additionally, although subtle changes, mostly involving Purkinje cells in the cerebellum, were identified in some day 19 chicken embryos, these neuropathological changes could not be linked to *in ovo* MeHg exposure.

9.2.2.3 Behavior

Neurobehavioral changes are one of the most serious effects to potentially result from MeHg exposure, as changes in ecologically relevant behaviors that impact survival and reproduction in individuals may result in population level effects. The studies in this dissertation are among the first to assess neurobehavior in developmentally exposed birds. Behaviors were assessed in day 1 and day 1 chicken hatchlings that had been exposed to MeHgCl *in ovo*, and despite the fairly high levels of THg in chick brains, we found no consistent relationship between

brain THg and righting reflex, performance on a level and angled beam, or startle response. This is in contrast to several other studies that report neurobehavioral changes in birds with experimental MeHg exposure, which are summarized in Table 9.3. In the study with the design most similar to ours, and the only to involve developmental exposure, no effects on righting reflex but decreased response to a frightening stimulus were seen in common loon chicks from eggs injected with 2.9 μg/g MeHgCl (Kenow et al. 2011). This is a dose much lower than those used in our studies, but injection into loon eggs was performed at incubation day 4 rather than at day 0 as in our studies. Injections at this later timepoint cause considerably higher toxicity (Heinz et al. 2006), and in a study involving the later injection timepoint, the LC_{50} for the chicken (0.44 μg/g) (Heinz et al. 2009) is actually lower than the LC_{50} for the loon (1.78 μg/g) (Kenow et al. 2011). In another common loon study by the same group, dietary exposure to high doses reduced righting reflexes in chicks starting only at at 37 days of age (Kenow et al. 2010). The studies of great egrets and American kestrels that exhibited general behavioral changes and coordination deficits involved feeding of older chicks and adults. Likewise, behavioral changes have been observed in adult common loons in the field (Evers et al. 2008, Nocera et al. 2008). When the findings in this dissertation are considered alongside the existing literature, it appears that developmental MeHg exposure has a lesser impact on neurobehavior than does longer term dietary exposure.

9.2.3.4 Potential Impacts of MeHg on Wild Embryos and Hatchlings

Methylmercury is generally considered to be of great neurotoxicological concern to developing embryos and young animals. Many studies have found associations between Hg exposure and neurochemical (Farina et al. 2003, O'Kusky et al. 1988) and behavioral (Baraldi et al. 2002, Carratu et al. 2006) alterations in rat embryos and pups. The studies described in this dissertation suggest that this may not be the case in birds as it is in mammals, as no consistent pathological, neurochemical, or neurobehavioral alterations could be attributed to MeHg exposure in these avian studies. Collectively, the work summarized in this dissertation and the existing literature (Table 9.1, Table 9.2, Table 9.3) have shown relationships between dietary MeHg exposure and neurological changes in juveniles and adults of several species, but very few controlled studies have shown similar effects with developmental exposure alone (Fig. 9.2). The contradictory findings in may result from differences in timing of brain development and maturation between birds and rodents (reviewed in Rostas et al. 1992). The lack of neurological impacts seen here may also stem from the plasticity and adaptability of the developing brain While known to be sensitive to toxicants, the embryonic brain is also highly adaptable and capable of repair following insult (Doi 2011, Ueno et al. 2006) and this adaptability may result in minimal effects in embryos and hatchlings. Mercury is also a toxicant that often displays a long latency period of up to several years before neurobehavioral changes become apparent in humans (Rice 1996, Spyker 1975). If such a latency period occurs in birds, the effects of *in ovo* exposure may not manifest until adulthood. Changes in GS, GAD (Rutkiewicz et al. 2011), and

the NMDA receptor (Rutkiewicz et al. 2011, Scheuhammer et al. 2008) were related to IHg but not OHg in wild bald eagles, but in embryonic birds the majority of the Hg is in the organic form (Chapter 4). Therefore a latency period may be attributed to slow demethylation of MeHg in the brain of embryos and hatchlings. Regardless of the reason for the apparent lack of neurological effects of developmental MeHg exposure, the work in this dissertation suggests that developmental exposure may not be of great neurological concern to wild embryos and hatchlings.

9.2.3 Limitations of Neurochemical Biomarkers

Neurochemical biomarkers have the potential to be applied as risk assessment tools, but work from this dissertation and other studies have drawn attention to limitations to their use in the field. First, the postmortem stability study demonstrated that while some glutamatergic and GABAergic neurochemical biomarkers are fairly stable under environmentally relevant postmortem and storage conditions (NMDA receptor, GAD activity), others are much less stable and might best be employed in studies where tissues can be freshly obtained and properly stored, or in studies of tissues with known and similar postmortem histories ($GABA_A$-benzodiazepine receptor, GS activity). A similar study has examined the stability of dopaminergic and cholinergic biomarkers under comparable storage conditions and has found that these biomarkers also vary in their stability (Stamler et al. 2005). While these two studies have not evaluated all possible postmortem conditions, collectively they propose a suite of stable neurochemical biomarkers from which researchers may choose for risk assessment

studies in wild birds. Although these works clearly demonstrate that there are limitations regarding biomarker stability to the use of some neurochemical biomarkers in wildlife tissues, they offer insight into ideal storage conditions to aid in study design and identify conditions under which some biomarkers are less stable to aid in data interpretation.

In addition to postmortem conditions, several other factors that may influence neurochemistry must be considered in studies involving neurochemical biomarkers. For example, neurochemical values often vary according to animal age (Kito et al., 1990), gender (Frick et al., 2002), and perhaps previously unstudied factors such as breeding status and seasonal variation that may be relevant in studies of wild animals. Matching samples for these variables whenever possible may help, but many of these factors are often unknown in wildlife studies, including the herring gull and bald eagle studies described in this dissertation. Therefore the findings from such studies must be interpreted cautiously. Because wild animals are exposed to a multitude of environmental stressors and contaminants, the lack of specificity of neurochemical biomarkers presents a significant limitation. Ideally, biomarkers of exposure or effects should be changes that can be related to the exposure or effects of a specific toxicant (Benford et al. 2000), but neurochemical changes can result from a variety of exposures. Lead (Pb) and manganese (Mn), in particular, are metals to which wild birds are exposed (Burger et al. 1999) and have been shown in the laboratory to induce changes in components of the glutamate and GABA neurotransmission pathways of rodents (Fitsanakis et al. 2005). In fact, correlations similar to those seen between Hg and GS and GAD in the bald eagle

study were also seen with Pb and other metals such as Cu, Zn, Co, and Cd in a subset of the same eagle samples (Nam et al. 2011). Though tissue Hg concentrations in these eagles were generally of more concern than were concentrations of other metals, many birds did exceed proposed thresholds for Pb toxicity. This highlights the fact that neurochemical biomarkers may not necessarily distinguish Hg or any other single chemical as a source of neurotoxicity, and also emphasizes the need for controlled laboratory studies to support field studies and to clarify the impacts of multiple toxicant exposures.

Finally, this dissertation leaves outstanding questions about the meaning and relevance of neurochemical changes. These types of changes are generally considered to be the earliest signs of neurological damage and can warn of toxicity before progression to structural and behavioral damage (Manzo et al. 1996). However, the work in this dissertation has not established the relationships between neurochemical change, brain lesions, and behavioral changes. Furthermore, meaning of the neurochemical changes is yet unknown, as many changes represent secondary adaptations intended to maintain homeostasis and minimize toxicity rather than direct insults (Siegel et al. 1999). Therefore interpretation of neurochemical findings remains difficult.

9.3 Future Research

Development of a threshold for neurological damage in wildlife is important if neurochemical biomarkers are to be used in risk assessment. The work presented in this dissertation along with other recently published studies (Table 9.1) looking

at biomarkers in birds with various levels of contaminants have helped establish that a threshold for neurochemical changes in juvenile and adult birds likely exists. A tentatively proposed threshold of approximately 5-10 μg/g d.w.was estimated using studies of only three species and a limited number of biomarkers, and could be greatly refined with additional studies of wild collected birds. Additional studies of herring gulls, bald eagles, and common loons with varying exposure levels could be valuable because these species are frequently studied as sentinels and our of concern for potential effects (Burgess et al. 2008, Evers et al. 2008, Fox et al. 2001), and species specific thresholds could help to more accurately assess their risks due to Hg. Studies of other species could also help to establish a more accurate broad threshold that can be applied across species. Studies of a larger number of neurochemical biomarkers could also help to identify which among neurochemical changes are most consistently associated with Hg and which are most sensitive and represent the earliest changes.

This dissertation has examined associations between brain Hg levels and neurochemical changes in birds, but to date no studies have demonstrated similar changes under controlled laboratory settings. Such studies are very important in order to establish a causal relationship between Hg exposure and neurochemistry in wild birds, as environmental factors and contaminants other than Hg (Nam et al. 2011) may have influenced the relationships that were seen between Hg and neurochemistry in bald eagles. Such studies can also complement wildlife studies that aim to identify thresholds for change. As studies presented in this dissertation have shown that Hg does not influence neurochemistry in embryos and hatchlings,

long term feeding studies in chicks and adults will be necessary to establish causality between Hg exposure and the changes in neurochemical biomarkers seen in wild eagles. These feeding studies could be useful not only for establishing the causal relationship between Hg and neurochemical changes, but they could also help to determine the yet unknown relationship between neurochemistry and behavior. Studies involving multiple chemical exposures could also be valuable for identifying potentially synergistic or antagonistic relationships with other neurotoxic contaminants to gain a better understanding of neurochemical impacts under true ecological conditions.

In this dissertation, few indications of neurological impacts were seen following developmental MeHg exposure in embryos and hatchlings. Though four species of birds were studied, previous egg injection work has shown that toxicity can vary greatly between species (Heinz et al. 2009). A similar study involving assessment of neurochemistry following MeHg egg injections in several species could help to confirm that developmental exposure does not have neurological impacts in birds, or it could help to identify species differences that might exist. Because mammalian studies indicate that impacts of MeHg are greatest with both developmental and postnatal exposure (Rice 1998, Spyker 1975), MeHg egg injection studies followed by post hatch feeding and assessment of neuropathology, neurochemistry, and neurobehavior should also be performed. In conjunction with feeding studies in chicks and adults, these studies could help to identify the exposures that place birds most at risk for the neurological effects seen in wild adults (Evers et al. 2008, Nocera et al. 2008).

Figures and Tables

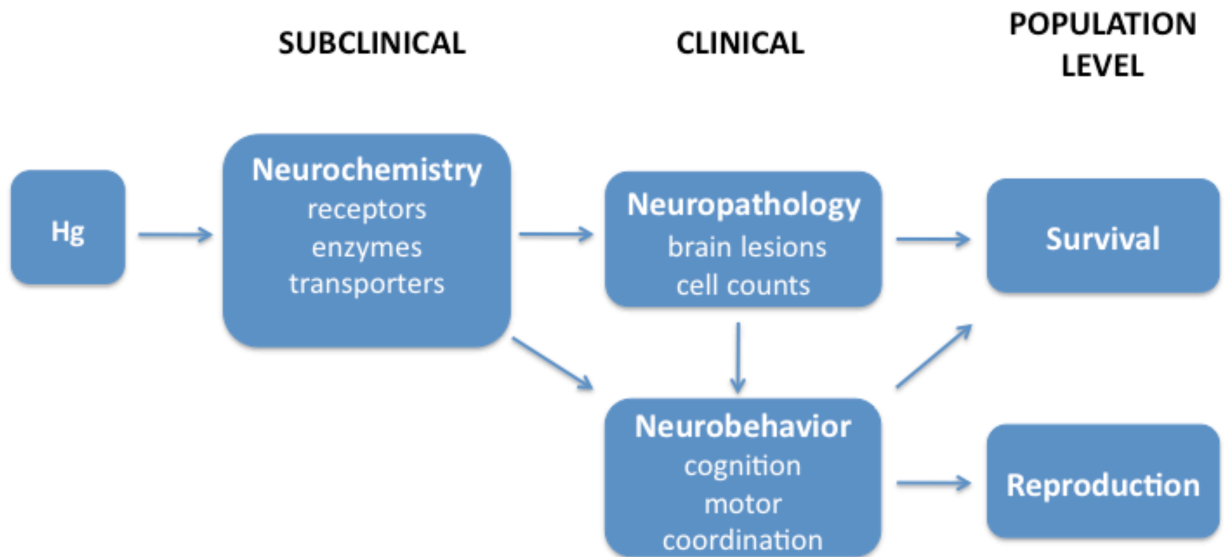

Figure 9.1 Schematic of the proposed progression and tiers of neurological change following exposure to mercury (Hg)

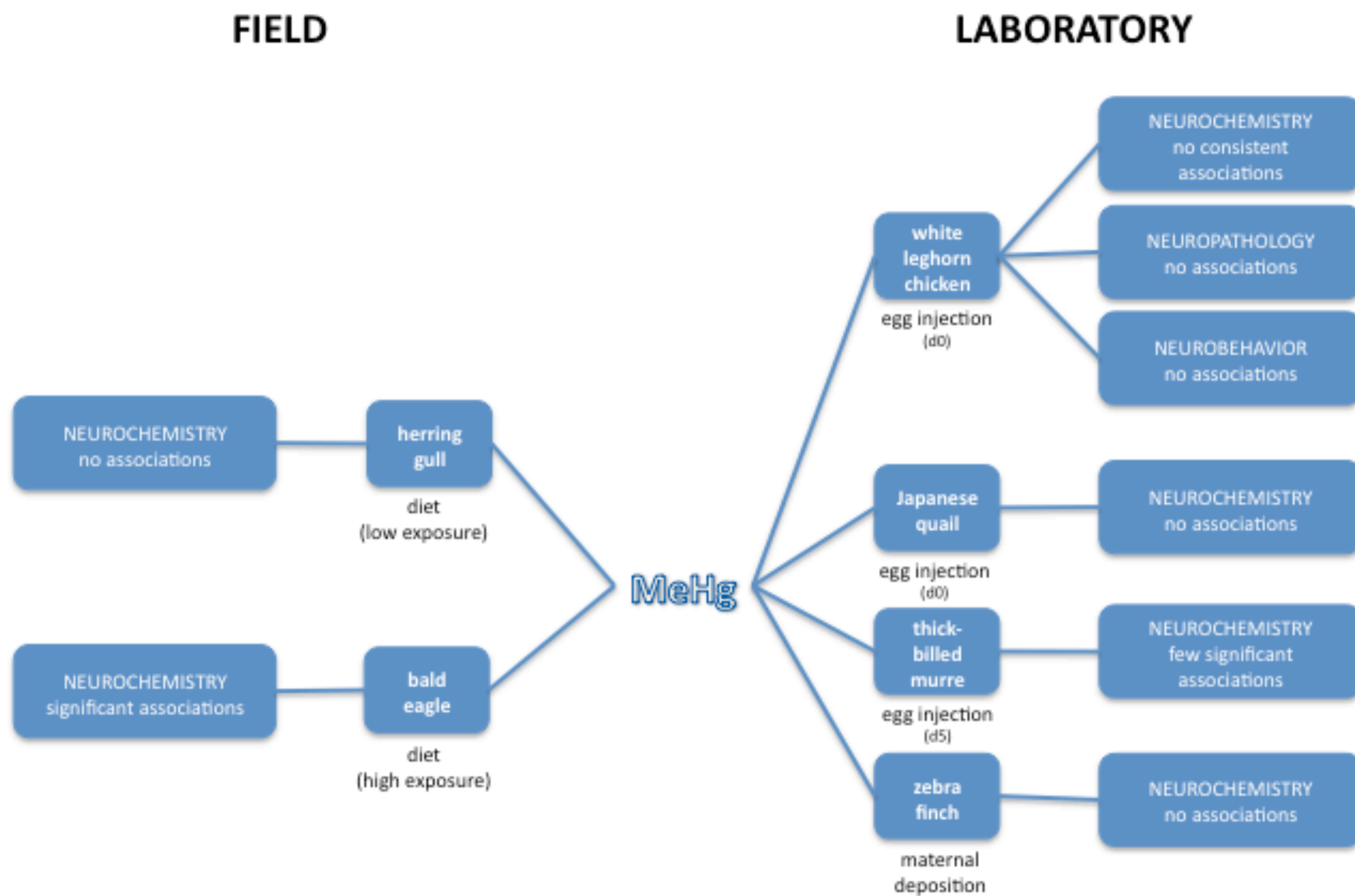

Figure 9.2 Summary of methylmercury (MeHg) exposure routes, avian species, and neurochemical findings from field and laboratory studies that are described in this dissertation

Table 9.1 Summary of published field studies investigating relationships between brain mercury (Hg) and neurochemistry in fish eating birds (THg=total mercury; MeHg=methylmercury; IHg=inorganic mercury)

Species	**Location of carcass collection**	**Age**	**Brain region studied**	**Range of brain THg concentrations (μg/g) d.w.**	**Neurochemical biomarkers assessed**	**Correlations with brain Hg**
bald eagle [1]	Canada	juvenile-adult	whole brain	0.3-23	ChE activity MAO activity NMDA receptor mACh receptor	NS NS - association (THg/IHg) + association (THg/MeHg)
bald eagle [2]	US Great Lakes	juvenile-adult	whole brain	0.2-34	GS activity GAD activity NMDA receptor GABA receptor	+ association (THg/IHg) - association (IHg) - association (THg/IHg) NS
common loon [1]	Canada	adult	whole brain	0.2-68	ChE activity MAO activity NMDA receptor mACh receptor	NS NS - association (THg/IHg) + association (THg/MeHg)
common loon [3]	Lake Erie, Canada	not reported	BS CBL CER	0.27-3.43 0.32-2.83 0.37-3.65	ChE activity MAO activity NMDA receptor mACh receptor	NS for any biomarker in any brain region
herring gull [4]	US Great Lakes	adult	CER	0.14-2.0	NMDA receptor mAChR nAChR	NS for any biomarker

[1] Scheuhammer et al. 2008
[2] Rutkiewicz et al. 2011
[3] Hamilton et al. 2011
[4] Rutkiewicz et al. 2010

Table 9.2 Summary of findings from studies in this dissertation investigating relationships between brain mercury (Hg) and neurochemistry in embryos and hatchlings following in ovo exposure to MeHg

Species	Age	Form of Hg	Exposure method	Brain region studied	Range of brain THg concentrations (μg/g) d.w. [a]	Correlations with brain THg (NS=not significant, +=positive, -+negative)			
						NMDA receptor	**$GABA_A$ receptor**	**GS**	**GAD**
chicken	embryonic day 19	MeHgCl	d0 air cell egg injection	CER CBL OL	0.01-21.8 [c]	NS (all regions)	NS (all regions)	NS (all regions)	NS (all regions)
chicken	embryonic day 10	MeHgCl	d0 air cell egg injection	whole brain	0.01-0.65 [b]	NS			
chicken	embryonic day 14	MeHgCl	d0 air cell egg injection	whole brain	0.02-3.6 [b]	NS	NS	NS	NS
chicken	embryonic day 19	MeHgCl	d0 air cell egg injection	whole brain	0.04-12.7[b]	NS	NS	NS	+ association
chicken	posthatch day 1	MeHgCl	d0 air cell egg injection	CER CBL OL	0.04-25.0 [c]	NS (all regions)	NS (all regions)	NS (all regions)	NS (all regions)
chicken	posthatch day 7	MeHgCl	d0 air cell egg injection	CER CBL OL	0.1-27.9 [c]	NS (all regions)	NS (all regions)	+ association (OL only) not reproduced	NS (all regions)
chicken	embryonic day 19	MeHgCys	d0 air cell egg injection	whole brain	0.01-6.0 [b]	+ association	- association	NS	NS
quail	embryonic day 15	MeHgCl	d0 air cell egg injection	whole brain	0.05-14.1 [b]	NS	NS	NS	NS
murre	pipping	MeHgCl	d5 air cell egg injection	whole brain	0.35-10.8 [b]	+ association	NS	NS	- association
finch	posthatch day	MeHgCys	maternal deposition	whole brain	0.03-37.5 [c]	NS	NS	NS	NS

[a] Values for chicken, murre, and finch studies were converted from w.w. to d.w. assuming 80% moisture; [b] Values presented are for whole brain; [c] Values presented are for cerebrum.

Table 9.3. Summary of laboratory studies investigating relationships between mercury exposure and behavior in birds

Species	Exposure type	Age tested	Behaviors observed	Behavioral outcomes
chicken [1]	Developmental (egg injection)	day 1 hatchlings day 7 hatchlings	level balance beam angled balance beam startle response righting reflex	No significant associations
common loon [2]	Developmental (egg injection)	hatchlings	righting reflex frightening stimulus response	Reduced response to frightening stimulus with highest treatment
common loon [3]	Dietary (dosed food) beginning at hatch	day 2 hatchlings- 15 week chicks	righting reflex ataxia time-activity budget frightening stimulus response parental call response	Reduced righting reflex in chicks older than 37 days with highest treatments
great egret [4]	Dietary (dosed food) beginning in day 12 chicks	10-14 week chicks	activity shade seeking hunting behavior	Ataxia at high dose, increased shade seeking behavior and decreased motivation to hunt at low dose by weeks 11-14
great egret [5]	Dietary (dosed food) beginning in day 12 chicks	10-13 week chicks	posture gait leg/wing reflexes feather cleanliness righting reflex	Ataxia at high dose, decreased feather cleanliness at low and high dose at week 10
American kestrel [6]	Dietary (dosed food) in adults for 59 days	adult	general toxicity	Impaired balance and coordination with high doses

[1] Rutkiewicz et al. (Chapter 5)
[2] Kenow et al. (2011)
[3] Kenow et al. (2010) .
[4] Bouton et al. (1999)
[5] Spalding et al. (2000)
[6] Bennett et al. (2009)

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