Interactions of Dietary Antioxidants and Methylmercury on Health Outcomes and Toxicodynamics: Evidence from Developmental Rat Model Studies and Human Epidemiology

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ABSTRACT

Methylmercury (MeHg) is an environmental contaminant that can cause neurotoxicity following developmental exposure even at minute levels. The primary source of MeHg exposure is via seafood consumption, which presents a difficult public health issue as seafood is also a rich source of nutrients. The characterization of the interaction between dietary nutrients and MeHg toxicity will enable improved MeHg risk assessment, and following additional research, may present a public health approach to reduce the risk associated with MeHg exposure without reducing the consumption of nutrient-rich seafood.

Since oxidative stress is considered to be one of the primary mechanisms underlying MeHg neurotoxicity, the interactions between antioxidant nutrients and MeHg is the focus of the present work. Firstly, we used an ethnobotanical study and laboratory assays to confirm the antioxidant activity and cultural appropriateness of Northern Labrador tea, *Rhododendron tomentosum* ssp. *subarcticum*, for the Canadian Inuit. Secondly, we determined the ability of *R.tomentosum* extracts (Tea) to ameliorate MeHg-induced toxicity in a rat perinatal exposure study. Developmental MeHg exposure (2 mg/KgBW/d for 6 weeks) was associated with delayed rat pup development and acoustic startle response, hyperactivity, decreased grip strength, and elevated brain N-methyl-D-aspartate receptor (NMDA-R) levels and serum lipid peroxidation. Surprisingly, Tea co-exposure (100 mg/KgBW/d for 6 weeks) modulated MeHg’s effects on brain NMDA-R levels and serum lipid peroxidation, but it also increased mercury serum concentrations. Thirdly, we used a toxicogenomics approach to explore the underlying mechanisms of MeHg neurotoxicity, and the ability of the Tea to
protect these molecular targets. MeHg exposure caused the down-regulation of Nr4a2 and its protein product Nurr1, which are implicated in developmental learning functions. Interestingly, the targeted functional groups and pathways included translation, cell cycle and cell division, which were not associated with oxidative stress defense or glutamate balance, the proposed primary mechanisms of neurotoxicity. These perturbations were not detected with MeHg + Tea co-exposure, suggesting the ability of Tea to reduce MeHg effects on these endpoints. Lastly, we investigated if we could detect the interaction of dietary nutrients on MeHg toxicokinetics in a human population. Costa Rica is a small Central American country with multiple sources of Hg and a high per capital fish consumption. Here we conducted a risk assessment survey of MeHg intake in 14 different population locales throughout the country. Of these 14 populations, 5 exceeded the World Health Organization’s recommended MeHg provisional tolerable daily intake (pTDI) of 0.2 µg/KgBW/d. This level of MeHg exposure was then confirmed with a cross-sectional epidemiological study in Heredia, where the pTDI was exceeded by 34% of woman participants largely from the consumption of canned tuna. Perhaps most interestingly, we detected that Hg body burden was significantly reduced by the consumption of antioxidant-rich dietary items. Based on our animal exposure and epidemiological study results, we hypothesized that MeHg toxicokinetics may be altered by dietary nutrients at the site of intestinal absorption from a disruption of gut flora, or at the site of cellular demethylation in tissues from the improvement of cellular redox state. The interaction of dietary nutrients on MeHg outcomes has a large impact on risk assessment and may provide a public health
approach for managing the risk associated with MeHg exposure without reducing local fish consumption.
RÉSUMÉ

Le méthylmercure (MeHg) est un contaminant environnemental qui peut provoquer la neurotoxicité développementale même après une exposition à des niveaux infimes. La principale source d'exposition humaine au MeHg est la consommation de produits de la mer, ce qui représente un difficile problème de santé public puisque ces produits sont aussi une riche source de nutriments. La caractérisation des interactions entre les nutriments alimentaires et la toxicité du MeHg permettra une meilleure évaluation des risques du MeHg, et après des recherches supplémentaires, pourrait représenter une approche de santé publique pour réduire les risques associés à l'exposition au MeHg sans réduire la consommation de produits de la mer riche en éléments nutritifs. Puisque le stress oxydatif est considéré comme l'un des principaux mécanismes qui sous-tendent la neurotoxicité du MeHg, les interactions entre les nutriments antioxydants et le MeHg sont l'objet de la présente étude. Tout d'abord, nous avons réalisé une étude ethnobotanique et des essais en laboratoire pour confirmer l’activité antioxydante et la pertinence culturelle du Thé du Nord Labrador, *Rhododendron tomentosum subarticum*, pour les Inuits du Canada. Deuxièmement, nous avons déterminé la capacité d’un extrait de *R.tomentosum* (Thé) à modifier la toxicité induite par le MeHg lors d’une exposition périnatale chez le rat. L'exposition au MeHg (2 mg/KgMC/j pour 6 semaines) a été associée à un retardement du développement et de la réaction de sursaut acoustique des ratons, à de l’hyperactivité, à une baisse de la force d’agrippement et à une élévation des niveaux du récepteur cérébral N-méthyl-D-aspartate (NMDA-R) et de la peroxydation des lipides sériques. Étonnamment,
la co-exposition avec le Thé (100 mg/KgMC/j pour 6 semaines) a modulé les effets du MeHg sur les niveaux de NMDA-R cérébraux et sur la peroxydation des lipides sériques, mais elle a aussi donné lieu à une ouverture précoce des yeux et à une augmentation des concentrations sériques de mercure. Troisièmement, nous avons utilisé une approche toxicogénomique pour explorer les mécanismes sous-jacents à la neurotoxicité du MeHg et à la capacité du thé à protéger ces cibles moléculaires. L'exposition au MeHg a causé la sous-expression de Nr4a2 et de son produit protéique Nurr1, qui sont impliqués dans le développement des fonctions d'apprentissage. Fait intéressant, les cibles et les voies moléculaires affectées par le MeHg comprenaient la traduction des protéines, le cycle cellulaire et la division cellulaire, des voies qui ne sont pas associées à la défense contre le stress oxydatif et le déséquilibre en glutamate, les principaux mécanismes proposés pour expliquer la neurotoxicité du MeHg. Ces perturbations n'ont pas été détectées suite à la co-exposition du MeHg avec le Thé, ce qui suggère que le Thé puisse réduire les effets MeHg sur ces paramètres. Finalement, nous avons cherché à savoir si nous pouvions détecter les interactions de nutriments alimentaires avec la pharmacocinétique du MeHg chez une population humaine. Le Costa Rica est un petit pays d'Amérique centrale présentant de multiples sources de mercure et une consommation élevée de poisson par habitant. Nous y avons conduit un sondage sur l'exposition au MeHg chez 14 populations locales à travers le pays. De ces 14 populations, 5 dépassent la dose journalière admissible provisoire (pTDI) de 0,2 µg MeHg/KgMC/j émise par l’Organisation Mondiale de la Santé. Ce niveau d'exposition au MeHg a ensuite été confirmé par une étude épidémiologique transversale à Heredia, où
la pTDI est dépassée par 34% des participantes, ce qui est principalement attribuable à la consommation de thon en conserve. Le plus intéressant est que nous avons détecté que la charge corporelle de mercure a été significativement réduite par la consommation d'aliments des riches en antioxydants. En se basant sur nos études animales et épidemiologiques, nous émettons l'hypothèse que la toxicocinétique du MeHg peut être altérée par les nutriments alimentaires au site d’absorption intestinale par une altération de la flore intestinale, ou au site cellulaire de déméthylation par une amélioration de l’équilibre oxydo-reductif. L’interaction des nutriments alimentaires sur les conséquences du MeHg a un grand impact sur l’évaluation des risques et pourrait fournir des approches de santé publique pour gérer les risques associés à l’exposition au MeHg sans réduire la consommation de poissons locaux.
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LIST OF ABBREVIATIONS

°C – degrees Celsius
μg – micrograms
μl – microlitre
μM – micromolar
-SH – sulphhydryl/thiol group
-SeH – selenol group
ANOVA – one way analysis of variance
BLAST – Basic Local Alignment Search Tool
BROD – benzylxoyresorufin-O-dealkylase
CAREG – Advanced Research in Environmental Genomics
cDNA – copy deoxyribonucleic acid
CECUNA – Centre for Ethical Conduct at the Universidad Nacional
Ci – curie
CNS – central nervous system
CO₂ – carbon dioxide
d – day
DAVID – Database for Annotation, Visualization, and Integrated Discovery
DNA – deoxyribonucleic acid
DPPH – 1,1-diphenyl-2-picrylhydrazyl
DW – dry weight of plant material
EROD – ethoxyresorufin-O-deethylase
EST – expression sequence tag
EtOH – ethanol
Fig – figure
fmol – femtomole
G – centrifugational force
GD – gestational day
GO – gene ontology
GSH – reduced glutathione
GSSG – oxidized glutathione
H⁺ – hydrogen ion
HCl – hydrogen chloride
Hg – mercury
HPLC-DAD – high performance liquid chromatography with diode array detector
IC₅₀ – inhibitory concentration at 50%
IgG – immunoglobulin G
KCl – potassium chloride
KEGG – Kyoto Encyclopedia of Genes and Genomes
KgBW – kilogram body weight
LAQAT – Atmospheric Chemistry Laboratory
LPS – Lipopolysaccharide
MeHg – methylmercury
MeOH – methanol
min – minute
ml – millilitre
mM – millimolar
mRNA – messenger ribonucleic acid
NCBI – National Center for Biotechnology and Information
NF-κB – nuclear factor kappa-light-chain-enhancer of activated B cells
nm – nanometer
nmol – nanomolar
NMDA-R – N-methyl-D-aspartate receptor
PCA – principal component analysis
PD – Parkinson’s Disease
PND – postnatal day
ppb – parts per billion
PPI – prepulse inhibition
ppm – parts per million
PROD – pentoxyresorufin-O-dealkylase
pTDI – provisional tolerable daily intake
PUFA – polyunsaturated fatty acids
RNA – ribonucleic acid
ROS – reactive oxygen species
rpm – rotations per minute
RT-PCR – reverse transcriptase polymerase chain reaction
RPMI – Roswell Park Memorial Institute
SD – standard deviation
Se – selenium
SEM – standard error of the mean
SPSS – statistical program for the social sciences
T2D – type 2 diabetes
TBARS – thiobarbituric acid reactive substances
Tea – Northern Labrador Tea (Rhododendron tomentosum ssp. subarcticum) extract
THg – total mercury
THP-1 – Human acute monocytic leukemia cell line
TNF-α – tumour necrosis factor-alpha
VIP – vasoactive intestinal peptide
WHO – World Health Organization
wk – week
CHAPTER ONE

General Introduction to Developmental Methylmercury Exposure and Nutrient Interactions

1.1 MeHg exposure as a worldwide concern

Methylmercury (MeHg) has been a well known public health issue since the severe poisoning of a Japanese fish-eating population in Minamata in the 1950’s. More recently, the role of dietary nutrients in modifying MeHg toxicity has been explored due to reports of interactions in various epidemiological studies (Chapman and Chan, 2000; Passos et al., 2007; Grandjean et al., 1992). Furthermore, a participant’s nutrient status is now recognized as a major confounding variable in MeHg exposure studies (Choi et al., 2008), and must be carefully considered for accurate risk assessment (NRC, 2000).

MeHg is an organic form of mercury (Hg) produced by the biotic methylation of mercury (Boening, 2000). There are multiple sources of both anthropogenic and naturally occurring Hg in our environment. The major anthropogenic source of Hg to the environment is the combustion of fossil fuels, primarily coal, in utility and industrial boilers, which accounts for approximately 2/3 of total global emissions (Pacyna et al., 2006). Other contributors to Hg emissions include the combustion of natural resources (biomass, coal and oil products), industrial production (cement, lead, zinc, steel, caustic soda via chlor-alkali processes, mercury, artisanal gold), and waste disposal of mercury containing
manufactured products (batteries, computer monitors, compact fluorescent light bulbs, measurement instruments, electrical switches) (Pacyna et al., 2006). There are also natural sources of Hg that significantly contribute to emission inventories such as volcanic activity and the release of geologically present Hg in soils and bedrock (Pacyna et al., 2006). In the aquatic environment, Hg can be methylated into MeHg, the form of Hg capable of biomagnification, which can result in substantially higher Hg concentrations in each trophic level. Therefore, MeHg concentrations are typically highest in top food-web predators, such as carnivorous fish, sea mammals, polar bears and sea birds (Campbell et al., 2005; Poissant et al., 2008; NRC, 2000). MeHg exposure in human populations primarily depends on the dietary habits of the consumer and seafood concentrations (NRC, 2000). MeHg concentrations in fish are expected to continue to increase throughout the world due to increased Hg emissions to the environment from anthropogenic activities (Fitzgerald and Clarkson 1991; Hammerschmid and Fitzgerald, 2006).

Ideally, MeHg contamination in our environment would be reduced through the regulation of Hg sales, use, emissions, and disposal. Indeed, international organizations have successfully reduced the use of Hg for health care (Practice Green Health, 2010) and artisanal gold mining (Global Mercury Project, 2010). However, MeHg contamination continues to be a public health issue that requires a more immediate response. Fish is a highly nutritious dietary source of proteins, essential fatty acids and macronutrients, and is often a primary food source for multiple populations throughout the world. A better understanding of the interactions of dietary nutrients and MeHg will improve health risk
assessments and may provide a crucial aspect of public health guidelines to alleviate the negative health outcomes associated with MeHg exposure while maintaining fish consumption. The aim of this doctoral thesis was to characterize the role of dietary antioxidants on MeHg toxicity and body burden.

1.2 Health outcomes from developmental MeHg exposure

The poisoning event of Minamata, Japan highlighted the most insidious property of MeHg; the neurotoxicity following developmental exposure. Infants exposed to MeHg through the placenta of exposed mothers, exhibited cerebral palsy-like symptoms even in the absence of indications of maternal poisoning (Marsh et al., 1980). Individuals affected by developmental MeHg exposure, often referred to as Minamata disease, typically exhibit peripheral dysesthesia (dysfunctional sense of touch), dysarthria (speech disorder), cerebellar ataxia (lack of voluntary motor coordination), tremor, visual field constriction, microcephaly, blindness, deafness, and gross impairment of motor and mental development (Igata, 1993). The average maternal hair concentration associated with these effects was estimated to be 41 µg/g (Akagi et al., 1998). The acute poisoning event in Iraq in 1971-1972 that resulted from the consumption of grains treated with a fungicide containing MeHg, confirmed these findings and established the first dose-response curve (Marsh et al., 1981). Although exposures throughout the world are considerably lower than those producing the historical epidemics of Minamata disease, there is growing evidence that chronic low-level exposure of MeHg also is cause for public health concern. For example, a recent study observed an inverse relationship between low-level prenatal MeHg exposure and visual
recognition performance of infants (Oken et al., 2005). The most startling result from this report was the detection of negative effects at maternal hair concentrations consistent with levels commonly measured in background US populations.

Two large cohort studies have contributed the majority of our current knowledge on chronic developmental MeHg exposure outcomes; one conducted in the Seychelles Islands of the Indian Ocean and the other in the Faroe Islands of the North Atlantic Ocean. The Seychelles Island population is exposed to MeHg primarily through the consumption of oceanic fish, whereas the Faroe Island population is exposed from marine mammal (pilot whale) consumption (Myers and Davidson, 2008). Interestingly, the Seychelles children tested at five and a half years of age had improved neurodevelopment scores associated with the increased maternal Hg-hair concentrations, a proxy indication of maternal fish consumption (Myers et al., 1997). Conversely, the Faroese children consistently demonstrated various neurobehavioral deficits at multiple ages of testing that were associated with developmental exposure (Grandjean et al., 1997; Debes et al., 2006). More recently there has been a convergence of the conclusions of these two major cohort studies, as the Seychelles children tested at 9 years of age also exhibited significant neurodevelopmental perturbations associated with maternal hair Hg exceeding 10 µg/g (van Wijngaarden et al., 2006).

Additional epidemiological studies have confirmed the neurotoxic properties of developmental MeHg exposure in French Guiana (Cordier et al., 2002), Madeira (Murata et al., 1999), Brazil (Grandjean et al., 1999), Canada (Saint-Amour et al., 2006), New Zealand
(Crump et al., 1998), whereas others conducted in Peru (Marsh et al., 1995) and Japan (Murata et al., 2004) found no association. The most commonly reported health outcome of chronic low dose exposure to MeHg during development are decreased motor function, attention, visuospatial performance, language ability, coordination, and increased potential latencies (auditory and visual), and tendon reflexes (Castoldi et al., 2008). In summary, there is indisputable evidence that MeHg is a potent developmental neurotoxin even at low level, background exposures. However, the elucidation of a dose-response relationship has been complicated by multiple factors such as varying testing parameters, pattern of MeHg exposure, age of testing, genetic susceptibility, and co-exposure to other contaminants or nutrients present in the diet (NRC, 2000).

1.3 Human MeHg exposure

In light of the epidemiological data generated primarily from the two large cohort studies conducted in the Seychelles and Faroe Islands, Canada has issued a provisional tolerable daily intake (pTDI) for women of reproductive age and children; 0.2 µg/KgBW/d (Health Canada, 2007a). The pTDI for the rest of the population is 0.47 µg/KgBW/d, reflecting the critical sensitivity of developmental MeHg exposure. The World Health Organization (WHO) promotes the same pTDI, as well as a maternal hair Hg concentration limit of 2 ppm (2 µg/g) (WHO, 2003). Over the past 10 years the recommended limits for MeHg consumption have consistently been lowered as new data surfaces, and recently it has been suggested that there may not be a safety threshold for adverse neurotoxicity from MeHg exposure (Rice, 2004). Since populations are primarily exposed to MeHg from the
consumption of fish and seafood, studies have focused on those who have high fish consumption or consume fish with high Hg concentrations.

For the majority of the populations worldwide the consumption of fish and seafood is the primary route of MeHg exposure, however MeHg may also be present in drinking water, cereals, vegetables, and meats (Galal-Gorchev, 1991). Therefore the MeHg exposure of various populations is highly variable, depending on local contamination issues and dietary habits. In Canada, extensive biomonitoring studies have focused on Aboriginal populations, which comprise Inuit, Métis, and First Nations peoples, due to the contamination of their traditional food (Berti et al., 1998; Arnold et al., 2003). The traditional diet of these peoples includes fish, seafood, and sea mammals, which can contribute to high MeHg exposure (Belanger et al., 2006). Developmental MeHg exposure from traditional food has been associated with negative health outcomes within an Inuit population (Saint-Amour et al., 2006). This presents a difficult situation as Aboriginal populations perceive their traditional food as an integral component of health and spiritual well-being. In addition, often in the smaller more isolated communities, traditional food provides a large portion of the population’s dietary nutrients as store bought alternatives are expensive and not readily available (Arnold et al., 2003). Clearly, the cultural, nutritional, social, economic and spiritual benefits of a traditional diet must be carefully weighed against the risk associated with elevated MeHg exposure. Alternative methods than those including the reduction of traditional food consumption should be considered for reducing MeHg exposure risk within these populations.
1.4 Nutrient-MeHg interactions

The ability of nutrients to modify the effects of developmental MeHg exposure has recently gained interest. The majority of research has focused on nutrients found in fish such as polyunsaturated fatty acids (PUFA) and selenium (Se), nutrients essential for optimal brain development (Chapman and Chan, 2000). PUFAs are required for cellular membrane composition, receptor binding efficiency, cellular interactions, and nutrient transport (Bourre et al., 1989). Se is an essential micronutrient with a narrow therapeutic range (Rayman, 2000). Se is required for the production of selenoproteins, which are crucial for the production of thyroid hormones and the major endogenous antioxidant system (Rayman, 2000). MeHg has an extremely high binding affinity for Se, able to irreversible inhibit Se-dependent enzymes and sequester Se (Boening, 2000). As a MeHg-Se conjugate, MeHg is less toxic to biological systems although malnutrition has been reported due to Se deficiencies following MeHg exposure (Watanabe et al., 1999; Dufault et al., 2009). The ability of various nutrients to interact on MeHg exposure outcomes has been demonstrated in multiple scenarios, highlighting the importance of understanding nutrient interactions in the risk assessment of MeHg exposure (Choi et al., 2008).

Although MeHg exposure primarily comes from “fish” not all types of fish have the same levels of MeHg or nutrients present. Typically, long-lived large carnivorous fish such as tuna, marlin, and swordfish, have higher MeHg concentrations than other fish due to bioaccumulation and biomagnifications (US FDA, 2009). Similarly, not all fish have the same concentrations of nutrients such as PUFAs and selenium, and interestingly long-lived
carnivorous fish are generally considered to be poor sources of these nutrients (Newland et al., 2008). This variation of MeHg and nutrient concentrations in fish allows for more accurate risk assessment in separating confounding effects (Choi et al., 2008), and provides the basic principle behind public health strategies that promote fish species with low MeHg and high nutrients (Burger et al., 2005).

Unfortunately, the majority of human health studies on seafood consumption have focused either on the risk of MeHg exposure or the benefits of nutrient intake, but not both simultaneously. It has been suggested that the incongruent outcomes of the two major developmental MeHg exposure cohort studies may be due to different dietary habits and inadequate information for negative confounding correction (Myers and Davidson, 1998). However, in both of the Faroe Island cohorts the adverse neurological function associated with developmental MeHg exposure was not influenced by PUFAs or selenium (Steuerwald et al., 2000).

Although PUFAs and selenium been the focus of the majority of nutrient-MeHg interaction research due to their presence in MeHg-contaminated fish, other dietary factors may have an effect on MeHg neurotoxicity. Without the collection of detailed dietary intake information of populations enrolled in developmental MeHg studies, it remains impossible to determine additional nutrient-MeHg interactions. However, a study conducted in the Brazilian Amazon with 26 women found a reduction of Hg body burden associated with the consumption of tropical fruit regardless of the level of fish consumption (Passos et al., 2003). This association was later confirmed with 449 participants from the same population,
which may suggest a method for risk reduction without disturbing the populations’ reliance on fish (Passos et al., 2007). Further evidence to support nutritional interactions primarily derive from animal model studies where laboratory environment and nutritional intake can be finely controlled. Nutrients may interact at various levels, including MeHg absorption, metabolism, molecular targets, or excretion.

1.5 MeHg Toxicokinetics

A large proportion (90-100%) of ingested MeHg is absorbed through the gastrointestinal track and enters the blood stream (WHO, 1990). In the blood stream 95% of the absorbed MeHg is found within red blood cells and the remaining 5% is bound to the sulfhydryl (-SH) groups of plasma proteins (Clarkson et al., 2007), and is distributed to all tissues in just over 24 hours (Carrier et al., 2001). The small fraction of MeHg that is not bound to red blood cells can form conjugates with L-cysteine, which structurally resemble methionine (Clarkson et al., 2007). MeHg-cysteine conjugates are transported on neutral amino acid carriers, such as LAT1 and LAT2, allowing MeHg to pass cellular membranes, including the blood brain and placental barriers (Simmons-Willis et al., 2002). Once MeHg crosses the placental barrier it accumulates in the rapidly developing tissues of the fetus, effectively clearing the maternal blood load and resulting in MeHg concentrations that are typically 6 times higher in fetal brain tissues than maternal blood (Cernichiari et al., 1995).

MeHg has a high affinity for sulfhydryl groups (-SH) and selenols (-SeH), which form part of cysteine and selenocysteine amino acids, and the active site of glutathione
Glutathione exists in a reduced form (GSH), where it is able to donate an electron to reactive oxygen species (ROS), and is converted to its oxidized form (GSSG). GSH-peroxidase and GSH-reductase enzymes maintain the balance of GSH:GSSG in healthy tissues. The sulfhydryl group of GSH also allows it to form conjugates with xenobiotics, including MeHg, aiding in cellular detoxification (Rooney, 2007). Once MeHg enters the cell with L-cysteine a large proportion of it immediately binds to abundant intracellular GSH compounds (Falinoga and Tusek-Znidaric, 2007). MeHg-GSH can then be exported back into extracellular space on endogenous GSH carriers and cycled back into the blood stream.

Within the cell, a portion of MeHg may be demethylated into Hg++, the form of Hg known to accumulate in tissues due to its poor mobility primarily bound as Se-Hg complexes (Clarkson et al., 2007). Indeed, the cellular transport mechanisms of MeHg as conjugates with L-cysteine and GSH account for the high mobility of MeHg within the body.

Only a small portion of circulating MeHg is subjected to urinary excretion as the majority of MeHg is eliminated from the body by the fecal route, with a half-life of approximately 50 days (WHO, 1990). Bacteria in the intestinal track demethylate MeHg into inorganic Hg, which is poorly absorbed and eliminated. However the majority of the MeHg undergoes extensive enterohepatic cycling. When MeHg-GSH passes through the liver a portion of it is deposited into the bile, where it is hydrolyzed into MeHg-cysteine complexes (Ballatori and Clarkson, 1982). When the bile is excreted into the intestines the MeHg-cysteine may undergo demethylation and fecal excretion however the majority of the MeHg is reabsorbed by the portal circulation, enters hepatic cells where it is bound to GSH, and re-
enters the blood stream to complete the enterohepatic cycle (Dutczak and Ballatori, 1992). Suckling rats do not produce GSH, which may be a factor in the relatively high accumulation of MeHg in developing tissues in utero (Ballatori and Clarkson, 1982).

The hair follicle has a high demand for amino acids for the production of keratins. The large neutral amino acid carriers present here allow MeHg-cysteine complexes to incorporate MeHg into the hair at a concentration approximately 250 times that in blood (Budtz-Jorgensen et al., 2004; Clarkson et al., 2007). Although MeHg bound to hair constitutes an inefficient method of elimination, the consistent blood-hair ratio is an excellent biomarker for MeHg exposure in epidemiological studies. Furthermore, the level of MeHg in maternal hair has a high degree of correlation to the level of MeHg in the brain of newborn infants (Cernichiari et al., 1995).

1.6 Animal model subject

Although the value of epidemiology for MeHg risk assessment is indisputable, the use of animal models is also an important source of information. Studies on human populations are inevitably correlative and rarely provide more than statistical control over dosing, nutrition, and environment, variables particularly influential on the neurotoxicity of chronic MeHg exposure. Studies involving animal model subjects provide a tight regulation of these variables, allowing the detection of subtle alterations and the elucidation of the mechanisms of MeHg toxicity. Behavioural studies on animal subjects provide an important link between human exposure outcomes and mechanistic studies.
To extrapolate meaningful information from animal MeHg exposure studies for the use of human risk assessment, it is important to account for their differences in physiology, neurodevelopment, and metabolism. In the case of rats as an animal model for developmental MeHg exposure studies, one of the most significant differences compared to humans is the timing of brain development, where the first 10 postnatal days in rats correspond to the last trimester of pregnancy in humans (Rice and Barone, 2000). Since MeHg is more efficiently transferred in utero than through lactation, the timing of the delivered dose to the brain varies between species. The circulation and compartmentalization of MeHg also varies between humans and rats. The ratio of MeHg in red blood cells to plasma (humans: 20, rats: 300) and blood to brain (humans: 6.0, rats: 0.1), as well as the MeHg excretion half life (humans: 58 days, rats: 16 days) all contribute to the requirement of a 10-fold higher dose of MeHg to achieve similar brain Hg levels in rats as humans from the same exposure (Newland and Rasmussen, 2000; Reed et al., 2006). Infants who died from high levels of developmental MeHg exposure in Minamata had an average brain Hg concentration of 5 µg/g (Newland et al., 2008). Autopsy data from the Seychelles Island study indicate the average brain Hg concentration of infants was 0.2 µg/g, which was 0.015 times maternal hair levels (Cernichiari et al., 1995). Using this ratio, the Faroe Island infants would have an average brain concentration of 0.4 µg/g (Grandjean et al., 1998). By using such information it is possible to approximate human exposure scenarios with appropriate animal model dosing levels.
The consequences of developmental MeHg exposure in rats are consistent with exposure in humans, including delayed developmental milestone achievement (Beyrouty and Chan, 2006), altered brain cytoarchitecture (Roda et al., 2008), and reduced motor function (Roegge and Schantz, 2006). Rats and humans also share the same MeHg-targeted regions within the CNS including the cerebellum and hippocampus, which result in learning and memory deficits (Roegge et al., 2006). The rat is quickly becoming a good model for toxicogenomic studies as the genome of the rat is now largely sequenced, and better annotated.

Rodent model exposure studies have provided further evidence of MeHg-nutrient interactions. The intestinal absorption of MeHg may be altered by dietary nutrients, particularly with reabsorption after the first pass of the enterohepatic cycle (Yannai and Sachs, 1993). For example, wheat bran fibre increases demethylation rates by the intestinal flora resulting in more efficient MeHg excretion in mice (Rowland et al., 1986), and on the other hand, reduced demethylation rates are observed following antibiotic treatment (Rowland et al., 1984). A diet enriched with milk proteins also demonstrated elevated MeHg reabsorption perhaps due to the disruption of the enterohepatic cycling with MeHg bound to proteins (Landry et al., 1979). In rats, the combination of dietary antioxidants, vitamin E and selenium, reduced developmental MeHg toxicity (Beyrouty and Chan, 2006). Although selenium has been the focus of the majority of nutrient-MeHg interaction studies, its protective effects is not reliably demonstrated (Chapman and Chan, 2000; Reed et al., 2006). Similarly, PUFAs and Se rich diets in in vivo experiments also did not provide any
considerable protection from MeHg, although some improvement in neuroperformance was noted (Paletz et al., 2006). There has been a number of recent papers demonstrating the ability of medicinal plant extracts to successfully ameliorate or attenuate MeHg induced neurotoxicity, including *Halimeda increassata* (Linares et al., 2004), *Polygala paniculata* (Farina et al., 2005) and *Cipura paludosa* (Lucena et al., 2007). It is interesting to note that all of these studies attributed the beneficial effects of these plant extracts to their antioxidant properties. However, these studies dosed adult animals and therefore do not constitute an adequate model for developmental MeHg exposure.

### 1.7 Mechanisms of MeHg toxicity

Multiple animal model studies have collectively provided insight into the molecular mechanisms underlying developmental MeHg neurotoxicity. Although several other potential molecular mechanisms of toxicity have been proposed, the majority of experimental evidence supports the role of oxidative stress and glutamate imbalance (Yin et al., 2007; Aschner et al., 2007; Shanker et al., 2005). Astrocytes, a major cell type within the CNS, are associated with both of these “housekeeping” neuronal functions. Furthermore, these cells are the site of MeHg accumulation (Aschner, 1996), and MeHg-induced toxicity appears to be secondary to astrocyte dysfunction (Morken et al., 2005). During development, these cells play a large role in neuronal migration, division and differentiation, and are also involved in the regulation of the blood-brain barrier (Aschner et al., 1999). As neurons are highly specialized electrical conductive cells they have minimal metabolic function, and instead rely on astrocytes for required compounds such as the
precursor molecules for GSH synthesis (Allen et al., 2002). Astrocytes also express multiple receptors and transporters, and are responsible for the tight regulation of synaptic neurotransmitter concentrations (Aschner et al., 1999). Details of both mechanisms are presented below, but ultimately they do not operate independently.

1.7.1 Oxidative stress

Oxidative stress is the disturbance of the pro-oxidant to antioxidant balance, and is implicated in the progression of multiple neurodegenerative diseases such as Alzheimer’s and Parkinson’s (Dorszewska et al., 2007). The CNS is particularly vulnerable to oxidative stress due to its high metabolic rate and disproportionately low levels of endogenous antioxidants (Ceccatelli et al., 2007). Furthermore, evidence suggests that oxidative stress insults that occurred during development increased sensitivities to such insults as the organism matures, thus it is possible for prenatal damage to become apparent only later in life (Ceccatelli et al., 2007). MeHg neurotoxicity is characterized by a long latency following exposure, where the effects of low dose chronic developmental exposure are often only apparent as the child begins to use higher-order cognitive functions that develop with maturity (van Wijngaarden et al., 2006).

Under normal conditions, reactive oxygen species (ROS) generated from toxic insult or metabolic functions are processed by antioxidant cellular systems, which include enzymatic reducing agents such as superoxide dismutase, glutathione peroxidase, and catalase, as well as non-enzymatic protein chelators, such as intracellular reduced
glutathione (GSH). ROS typically include oxygen radicals, such as superoxide, hydroxyl, and hydrogen peroxide, which are highly oxidizing and cause damage to proteins, DNA, lipids and other cellular macromolecules. If the oxidative stress persists, cells undergo apoptosis or necrotic cell death. The varying levels of endogenous antioxidants or sensitivity to ROS production in different cell populations can result in targeted damage (Kaur et al., 2007). For example, cerebellar granule cell neurons are extremely sensitive to even mild oxidative stress (Dare et al., 2000). Indeed, pathology reports confirm that cerebellar granule layer of the brain is the primary target of chronic MeHg poisoning in humans (Eto, 1997) and rats (Eto et al., 1997).

Oxidative stress has been implicated in multiple experimental models of MeHg neurotoxicity. Elevated ROS levels in the cerebellum have been found in MeHg exposed rats (Jie et al., 2007) and mice (Stringari et al., 2006). MeHg enters the cell and depolarizes the mitochondrial membrane, which uncouples the electron transport chain and releases ROS into the cytoplasm (Yee and Choi, 1996). MeHg also impairs GSH production by blocking cysteine uptake in astrocytes, inhibiting the cell’s antioxidant deference mechanisms ability to respond to the elevated ROS presence (Allen et al., 2002). Co-exposure studies have demonstrated the protective effects of antioxidants on MeHg-induced neurotoxicity. In rats, vitamin E and selenium supplementation largely mitigated developmental MeHg toxicity (Beyrouty and Chan, 2006). Antioxidants probucol, vitamin E, and propyl gallate were effective in MeHg treated rat cerebellar granule neuron cell cultures (Gasso et al., 2001). Ebselen, a potent hydrogen peroxide scavenger, also inhibited MeHg-induced toxicity and
oxidative stress in a dose dependant manner in cortical slices of mice brains (Farina et al., 2003). Another important implication of excessive ROS accumulation is the resultant interference of the EAAT1 glutamate transporter efficiency (Allen et al., 2001).

1.7.2 Glutamate imbalance

One of the main functions of astrocytes is the uptake of synaptic cleft neurotransmitters to ensure proper neuronal signalling. An excess of the excitatory neurotransmitters in the synaptic cleft causes excitotoxicity, characterized by prolonged depolarization of neurons, excessive intracellular calcium concentrations, ROS accumulation, and ultimately cell death (Choi, 1992). Glutamate is the principal excitatory neurotransmitter in the CNS, which activates four types of glutamate receptors, including the N-methyl-D-aspartate receptor (NMDA-R). NMDA-Rs are present in high concentrations in the cerebellum and hippocampus, and are crucial for neuronal plasticity, the underlying process of learning and memory (Scheetz and Constantine-Paton 1994). During fetal development NMDA-Rs are involved in the establishment of neuronal circuitry, which may be a contributing factor of the exquisite fetal sensitivity to neurotoxicants such as MeHg (Haberny et al., 2002).

MeHg inhibits the uptake of glutamate by astrocytes (Aschner et al., 1993) by blocking transporters such as EAAT1 (Allen et al., 2001). The excess glutamate within the synaptic cleft hyper-stimulates glutamate receptors, particularly NMDA-Rs, leading to post-synaptic neuron excitotoxicity (Yin et al., 2007). The role of NMDA-Rs in MeHg neurotoxicity
has been confirmed in various *in vivo* MeHg exposure studies. For example, MeHg can perturb the glutaminergic system long after the cessation of exposure (Cagiano et al., 1990), alter NMDA-R mRNA expression levels (Baraldi et al., 2002), and reduce NMDA-R binding (Basu et al., 2007).

MeHg co-exposure studies provide further evidence of the role of glutamate excitotoxicity and oxidative stress in MeHg neurotoxicity. MeHg-induced neurotoxicity can be mitigated by co-exposure with NMDA-R antagonists, such as MK-801 (Zhang et al., 2003; Faro et al., 2002; Juarez et al., 2005). Various purified phytochemicals or plant extracts have been shown to confer some protection against NMDA receptor agonist-induced excitotoxicity and oxidative stress *in vivo* and *in vitro* (Silva et al., 2008; Campos-Esparza et al., 2009; Chen et al., 2008; Cho and Lee, 2004) and interestingly, similar findings have been reported following co-exposure with MeHg (Lucena et al., 2007; Yongjin et al., 2008; Farina et al., 2005). Possible mechanisms involved in the mitigation of MeHg effects by phytochemicals may include the reduction of reactive oxygen species, activation of enzymatic antioxidant systems, restoration of the mitochondrial membrane potential, and modulation of cell signalling pathways (Campos-Esparza et al., 2009). Dietary phytochemicals may also affect additional endpoints such as MeHg bioavailability and pharmacokinetics (Passos et al., 2007; Laird et al., 2009).
1.8 Northern Labrador Tea

Northern Labrador Tea, *Rhododendron tomentosum* (Stokes) Harmaja ssp. *subarcticum* (Harmaja) G. Wallace, of the Ericaceae family, is a woody evergreen shrub endemic to the circumpolar subarctic (Fig. 1.1). *R. tomentosum* is extensively used within First Nations Populations as a medicinal plant for the treatment of type 2 diabetes (Fraser et al., 2007; Harbilas et al., 2009), respiratory illnesses (Black et al., 2008), and infection (Arnason et al., 1981). In laboratory settings this plant has demonstrated the ability to regulate glucose and insulin levels (Harbilas et al., 2009), to inhibit cytochrome P450s isoforms (Tam et al., 2009), and provide considerable cellular protection from oxidative stress (Fraser et al., 2007). Due to the role of oxidative stress with the etiology of developmental MeHg neurotoxicity and the cultural acceptance of this antioxidant-rich plant within Canadian Inuit populations, we decided to explore the possibility of mitigating MeHg neurotoxicity with an extract of *R. tomentosum*, herein referred to as “Tea”.

1.9 Hypothesis and objectives

The aim of this doctoral project was to characterize the interaction between dietary antioxidants and MeHg. Firstly, we conducted an ethnobotanical study to find a dietary source of antioxidants that was culturally relevant and readily available for Inuit in Nunavut, Canada, a population affected by MeHg contamination issues. We hypothesized that plants most commonly used by the Inuit for their health promoting properties would have high antioxidant properties. Secondly, we used a rat perinatal study to investigate the developmental, behavioural, and biochemical outcomes associated with MeHg exposure.
Figure 1.1  Northern Labrador Tea (*Rhododendron tomentosum* ssp. *subarcticum*) in Iqaluit, Nunavut in July 2006.
and MeHg + Tea co-exposure. We hypothesized that the antioxidant properties of \textit{R. tomentosum} would alleviate the MeHg-induced developmental neurotoxicity. Thirdly, we explored the molecular targets of developmental MeHg exposure and MeHg + Tea co-exposure using a toxicogenomic approach. We hypothesized that targeted molecular pathways would include glutamate transporters, NMDA receptor signaling, GSH systems and that MeHg + Tea co-exposed animals would demonstrate reduced perturbations in pathways related to the GSH system and other endogenous antioxidants. Lastly, we wanted to determine if we could detect interactions between dietary antioxidants and MeHg in a human population using a cross-sectional epidemiology study conducted in Costa Rica. We hypothesized that there would be a reduction in MeHg body burden associated with increased consumption of high antioxidant food items.

1.10 Thesis presentation

This doctoral thesis is a presentation of four manuscripts (one published, two submitted, and one in preparation), organized into four research chapters. Chapter two highlights the ethnobotany of Northern Labrador Tea as well as the characterization of the phytochemical constituents present in the extract used for animal dosing (Black et al, 2008; and manuscript submitted to \textit{Planta Medica}). Chapter three details the biochemical and neurobehavioral effects resulting from developmental MeHg exposure or MeHg + Tea co-exposure (manuscript submitted to \textit{Food and Chemical Toxicology}). Chapter four explores possible mechanisms and targeted pathways following developmental MeHg exposure or MeHg + Tea co-exposure using a toxicogenomic approach. Finally, chapter five expands to
include MeHg exposure risk assessment from fish consumption in various locations in Costa Rica, and an epidemiological study on nutrient-MeHg interactions in a Costa Rican population (manuscript in preparation).
CHAPTER TWO

Phytochemical and Ethnobotanical characterization of Northern Labrador Tea (*Rhododendron tomentosum* ssp. *subarcticum*)


And


1. Contributed to original ideas, oversaw plant material collection, prepared plant extract, measured plant DPPH antioxidant properties, data analysis, and manuscript preparation. *Department of Biology, University of Ottawa*.

2. Conducted HPLC analysis of plant extract. *Department of Biology, University of Ottawa*.

3. Collected plant material. *Nunavut Research Institute, Iqaluit, Nunavut, Canada*.

4. Assessed plant extract for TNF-α anti-inflammatory activity. *Department of Biology, University of Ottawa*.

5. Critical review of the manuscript. *Department of Pharmacology, University of Montreal*.

6. Provided ethnobotanical information and taxonomic verification of Labrador tea. *Biology Research Institute, University of Montreal*.

7. Contributed to original ideas, provided laboratory resources and revised the manuscript. *Department of Biology, University of Ottawa*.
2.1 Introduction

The use of traditional medicinal plants is widely practiced in North American aboriginal populations, with approximately 2500 plants identified (Moerman, 1996). The pairing of traditional approaches with modern medicine to treat illnesses in aboriginal populations often results in better compliance than using conventional medicine alone (Spoor et al., 2006). Illness treatment with botanicals represents a new challenge for care providers as the medicinal plants often lack characterization information or clinical support. This report focuses on the characterization and seasonal quantification of the phytochemical constituents of a plant commonly used as a traditional medicine by multiple First Nations populations including the Canadian Inuit. Additionally, the collection and documentation of traditional knowledge is an important part of the preservation of culture.

*Rhododendron tomentosum* (Stokes) Harmaja ssp. *subarcticum* (Harmaja) G. Wallace, of the Ericaceae family, is a woody evergreen shrub commonly called Northern Labrador Tea. *R. tomentosum* is endemic to the circumpolar subarctic with a short snow-free growing season and long daylight exposures. *R. tomentosum* is among the most commonly used medicinal plants in multiple Canadian First Nations populations for the treatment of type 2 diabetes (T2D) (Fraser et al., 2007; Harbilas et al., 2009), respiratory illnesses (Black et al., 2008), infection (Arnason et al., 1981), and other uses (Table 2.1). There is a strong consensus amongst populations for selected usage of *R. tomentosum* such as stomach ache, cold symptoms, and toothache (Table 2.1). Labrador Tea, *Rhododendron*
<table>
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<th>Names</th>
<th>Uses</th>
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<td>Tea; cold and flu symptoms; breathing; toothache; stomachache; fire fuel</td>
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<td>Qisiqtuut; Qijuktaaqpait</td>
<td>Fire fuel; sore throat and cough; tobacco; canker sores (in the mouth); headache; eye problems; dry skin</td>
<td>Wilson (1978); Black et al. (2008); Mallory &amp; Aiken (2004); Ootoova et al. (2001)</td>
</tr>
<tr>
<td>Inuit (Greenland)</td>
<td>Kajaussat</td>
<td>Tea</td>
<td>Le Mouël (1969)</td>
</tr>
<tr>
<td>Inuit (Greenland)</td>
<td>Tilaaqiuq</td>
<td>Tea</td>
<td>Jones (1983)</td>
</tr>
<tr>
<td>Inuit (Alaska; St. Lawrence; Western Canada); Haida; Nuxalk</td>
<td>Auyut</td>
<td>Tea (with medicinal value); stomachache; tuberculosis</td>
<td>Oswalt (1957); Young &amp; Hall (1969); Anderson (1939); Ager &amp; Ager (1980); Griffin (2001)</td>
</tr>
<tr>
<td>Gwich’in</td>
<td>La dee musket lidii masgit</td>
<td>Tea; colds and hangovers; overall energy; nasal congestion</td>
<td>Holloway &amp; Alexander (1990); Andre &amp; Fehr (2001)</td>
</tr>
<tr>
<td>Cree (James Bay)</td>
<td>Wiisichipkw-h</td>
<td>Wounds and skin sores; mouth sores; headaches; stomachache; coughs; sore throat; cold; tonic; aches and pains; flu</td>
<td>Marshall (2006)</td>
</tr>
<tr>
<td>Cree (Hudson Bay)</td>
<td>Uschiischipakw</td>
<td>Headache; blurred vision; appetite; diarrhea; abscesses; toothache; back and kidney pain; arthritis; infections; inflammation</td>
<td>Cuerrier et al. (in prep.)</td>
</tr>
</tbody>
</table>

*a Adapted from Black et al. 2011 (In press)*
*groenlandicum* (Oeder) Kron & Judd. has overlapping habitat in sub-boreal climes with Northern Labrador Tea, and is often used interchangeably with an occasional preference for using the smaller Northern Labrador Tea for children. These species differ in their phytochemical composition, at least in their volatile oil contents, with *R. groenlandicum* having more germacrone and *R. tomentosum*, more ledol (Marles et al., 2000). Populations today still use these plants as a topical treatment for toothaches or wounds, and as a tea for general consumption. *R. tomentosum* has demonstrated a variety of medicinal properties as a treatment for T2D, including glucose and insulin regulation, and oxidative stress protection (Harbilas et al., 2009; Fraser et al., 2007). In addition, *R. tomentosum* has moderate inhibitory activity on drug-metabolizing isoforms of cytochrome P450s (Tam et al., 2009). These results support the pharmacological activity of *R. tomentosum*, with the suggestion that further *in vivo* and *in vitro* investigations be undertaken (Fraser et al., 2007). Although other *Rhododendron* species have been phytochemically characterized (Harbourne & Williams, 1971; Louis et al., 2010), the composition of *R. tomentosum* remains largely unknown.

Multiple epidemiological and clinical studies have confirmed the health benefits resulting from antioxidant intake, such as decreased prevalence of cancer, improved memory function, increased physical endurance capacity, improved prognosis for T2D, and cardioprotection (*Review: Tomita et al., 2010; Kaneto et al., 2010*). The antioxidant and anti-inflammatory properties of medicinal plants are often attributed to their phenolic content (Bogani et al., 2007). Plant phenolic content has a high interspecies and intraspecies variation, highlighting the
importance of species and condition specific characterization. Although this is becoming a more common approach, information remains limited.

The phytochemical constituents of plants vary throughout the annual seasons due to genetic determinants and response to environmental conditions such as; herbivory, UV exposure, day length, temperature, and soil nutrients (Slimestad and Verheul, 2005; Tegelberg et al., 2002). The seasonal variation of phytochemicals present in plants of commercial importance is often well characterized to predict the timing of optimal harvest quality. In greenhouse-grown cherry tomatoes higher levels of phytochemical metabolites such as lycopene, ascorbic acid and β-carotene were present during the summer months, which were attributed to sunlight exposure (Slimestad and Verheul, 2005). Similarly in Australian-grown tea (Camellia sinensis), the main tea flavanols responsible for the quality of the product (epigallocatechin, epicatechin gallate and total catechin gallates) were highest during the summer months, which were attributed to temperature, sunlight exposure and/or day length (Yao et al., 2005). These seasonal trends may be particularly dramatic in high latitude arctic environments due to the importance of photoprotection in climes with a short summer season with long daylight hours (Grace et al., 2005).

When characterizing medicinal plants it is important to determine the optimal harvest time. The objectives of this study were to 1) quantify the seasonal variation of the phenolic compounds found in R. tomentosum leaf extract, 2) determine the seasonal variation of its antioxidant and anti-inflammatory activities, and 3) assess the relationship between phenolic content, antioxidant and anti-inflammatory activities, and environmental growing conditions.
We used HPLC/DAD to quantify 15 of the most abundant phenolic constituents. A 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging assay and TNF-α reduction in a LPS stimulated THP-1 monocyte assay were also used to characterize the plant’s antioxidant and anti-inflammatory properties respectively. It is hypothesized that the peak phenolic content would correspond to the longest daylight days of the season, which will in turn correspond to the peak antioxidant and anti-inflammatory activity.

2.2 Materials and Methods

2.2.1 Plant material collection and extraction

*Rhododendron tomentosum* ssp. *subarcticum* was collected bimonthly and in triplicate throughout the snow-free season from May to September 2006 near Iqaluit, Nunavut, Canada (N 63° 45.3’, W 68° 30.6’) according to traditional methods described by Inuit (Black et al., 2008; Ziegler et al., 2009; Mallory et al., 2004). The first 10 cm of the plant branch was removed and allowed to air dry in darkness for 24 hours. Dried plant material was ground with a Wiley Mill (A.H. Thomas Co., Swedesboro, NJ, USA) to a pore size of 2 mm and extracted twice in 80% ethanol (10 ml/g) for 24 hours. The solvent was evaporated, the homogenized extract was freeze dried and stored in amber vials at -20°C. Voucher specimens were deposited and taxonomically verified by Alain Cuerrier at the Marie-Victorin Herbarium of the Plant Biology Research Institute, University of Montreal (IQAL04-1A & B).

Observations on the plant’s life cycle (*i.e.* flowering, leave budding, *etc.*) were recorded at the time of sampling. Daily climatic information was collected (Environment Canada, 2010) and
daily sunlight hours (NRC, 2010). All values were reported as a two week average prior to the date of plant collection.

2.2.2 Chemicals and standards

(+)-Catechin, chlorogenic acid, para-coumaric acid, myricetin, quercetin, quercetin-3-O-galactoside, quercetin-3-O-glucoside, quercetin-3-O-rhamnoside, procyanidins, resveratrol, taxifolin (95% purity) were purchased from Chromadex Inc. as they represented a diverse selection of phenolic compounds (Irvine, CA, USA). HPLC grade water, acetonitrile and formic acid (99% purity) were purchased from Sigma-Aldrich, (Brockville, ON, Canada).

2.2.3 DPPH free radical scavenging assay

The free radical scavenging activity of the plant extract was measured with a 1,1-diphenyl-2-picrylhydrazyl (DPPH) colorimetric assay according to the method described previously (McCune and Johns, 2002), modified for use with a plate reader. Serial dilutions were prepared from a 2 mg/ml stock solution of each plant extract and ascorbic acid in MeOH. In a 96-well plate, 198 µl of 100 µM DPPH solution was added to 33 µl of extract in triplicate. Plates were incubated for 10 minutes at room temperature and the absorbance was read with a Beckman® DU320 Spectrophotometer at 517 nm. An inhibitory concentration at 50% (IC$_{50}$) was calculated for each extract (3 biological collection replicates for 8 collections, in triplicate) from the linear portion of the dose-response curve of ascorbic acid. For graphical purposes, the antioxidant activity was presented as $1/(IC_{50})$. 
2.2.4 TNF-α anti-inflammatory assay

The anti-inflammatory activity of the plant extract was assessed with a tumour necrosis factor-alpha (TNF-α) assay, where TNF-α production is measured in a human acute monocytic leukemia cell line (THP-1) following lipopolysaccharide (LPS) stimulation. THP-1 cells (ATCC, Manassas, VA, USA) were cultured in RPMI 1640 media (ATCC, Manassas, VA, USA) supplemented with 1% 0.05 mM beta-mercaptoethanol, 1% penstrep (Invitrogen, Mississauga, ON, Canada) and 10% fetal bovine serum (Invitrogen, Mississauga, ON, Canada), in a 37°C humidified environment with 5% CO₂. Cells were transferred (3x10⁴ cells/well) in 96-well plate, followed by the addition of plant extract dissolved in 80% EtOH for a final volume of 300 µl/well and a final EtOH concentration of 0.5%. Plant extracts were assayed at 100 µg/ml. Parthenolide (Sigma-Aldrich, St-Louis, MO, USA), a well known anti-inflammatory sesquiterpene lactone found in feverfew (Tanacetum parthenium), was used as a positive control at 10 µg/ml, and 0.5% EtOH was used as a vehicle control. All extracts and controls were assayed in triplicate. Following the addition of extracts and controls, cells were incubated for 2 hours, and then stimulated with 1 µg/ml LPS purified from Escherichia coli (Sigma-Aldrich, St-Louis, MO, USA) and allowed to incubate for 20 hours. An unstimulated control containing 0.5% EtOH but no LPS was also assayed. After incubation, cells were centrifuged at 2000 rpm (500 G) for 10 minutes at room temperature. Cell culture supernatants were separated and stored at -80°C for subsequent analysis. DuoSet® ELISA development kits (R & D Systems, Minneapolis, MN, USA) were used according to the manufacturer’s protocol. Raw TNF-α values (pg/ml) were transformed into the percent TNF-α response relative to the LPS-stimulated EtOH vehicle control (set as 100% TNF-α
production), thus extracts demonstrating the most anti-inflammatory activity have the lowest percent TNF-α response relative to the vehicle control. For graphical purposes, the anti-inflammatory activity is presented as 1/(percent TNF-α response relative to the vehicle control) for each plant extract (8 collections in triplicate).

2.2.5 High pressure chromatography

HPLC-DAD analysis was adapted from a previously described method (Saleem et al., 2010). Analyses were carried out on an Agilent 1100 series HPLC-DAD-APCI/MSD system (Agilent Technologies Inc., CA, USA). The system consisted of an online degasser, a quaternary pump (maximum pressure 400 bars), an auto sampler with a 100 μl built-in injection loop, a column thermostat compartment, a diode array detector with a flow cell (6 mm path length, maximum pressure 400 bars). The mobile phase consisted of 0.1% formic acid in water (solvent A) and acetonitrile (solvent B). A linear gradient of 10-55% B was applied over 25 min at a flow rate of 1.5 mL/min. The separations were performed on a Luna column (3μ C18(2) 100 A, Part# 00D-4251-B0, Serial#493611-1, Batch#5292-31, 100x2.0 mm) connected with a security guard (Phenomenex Inc. Torrance, CA, USA). The column was washed for 5 min with 100% B in isocratic conditions and equilibrated for 5 min. All extracts were dissolved at 40 mg/ml in 100% methanol and 1 μl was injected in triplicate. The elutions of target compounds were monitored at DAD wavelengths of 250 nm and 325 nm. The column oven temperature was maintained at 55°C. Calibration curves were constructed using Chemstation B.03.02 based on the area under the peaks by injecting authentic standards serially diluted within the concentration range that bracket the expected concentration in the plant extracts. The compounds with tentative
identification were quantified based on calibration curves of chemically similar authentic compound. Compounds were identified based on comparison to retention times and UV spectra of pure standards relative to a programmed library of known UV spectra, and further confirmed with mass spectrometry fragmentation patterns according to previously described methods (McIntyre et al., 2009). Standard compounds used to monitor extracts were (+)-catechin, chlorogenic acid, para-coumaric acid, myricetin, quercetin, quercetin-3-O-galactoside, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside.

2.2.6 Statistical Analysis

Seasonal variation of phytochemical quantities, antioxidant DPPH activity, and anti-inflammatory TNF-α response, were assessed for normality and distribution, and evaluated with one way analysis of variance (ANOVA) followed by the Tukey post-hoc test when appropriate. When required, data was log transformed. Total constituents were calculated as the sum of the quantities of the 15 characterized constituents. Two-tailed Pearson correlations were used to detect associations between seasonal data and medicinal properties. All analyses were performed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when $p < 0.05$.

2.3 Results

2.3.1 Seasonal variation of environmental conditions

The environmental conditions during the summer growing season of the *R. tomentosum* collections in Iqaluit, Nunavut are characterized by long daylight hours and a short growing
season (Table 2.2). *R. tomentosum* unfolds leaf buds in June soon after the snow cover melts and average minimum temperatures are above freezing. The longest day of the year, June 21, had 20.83 hours of sunlight (sunrise to sunset) and 3.17 hours of twilight at this latitude. Flowers are produced in July, the warmest month with an average high temperature of 15°C and a low of 5.2 °C. At the end of September the leaves of *R. tomentosum* begin to turn red in preparation for winter, when temperature lows can dip below freezing once again.

### 2.3.2 Seasonal variation of phytochemical constituents

Fifteen phytochemicals were identified and quantified in *R. tomentosum* leaf extract throughout their growing season (Fig. 2.1). HPLC/DAD with a detection at 325 nm was used to quantify chlorogenic acid, p-coumaric acid, myricetin, quercetin, quercetin-3-*O*-galactoside, quercetin-3-*O*--*O*-glucoside, quercetin-pentoside, quercetin-3-*O*--*O*-rhamnoside and three caffeic acid derivatives, and HPLC/DAD with a detection at 280 nm was used to quantify (+)-catechin and three procyanidins (Fig. 2.2).

Of the fifteen constituents quantified, (+)-catechin, quercetin-pentoside and quercetin-3-*O*--*O*-galactoside were the most abundant (Fig. 2.3). Firstly, (+)-catechin had a seasonal average of 6.75 mg/g DW (milligrams of extract per gram of plant dry weight) and range of 3.21 – 10.2 mg/g DW. Quercetin-pentoside had a seasonal average of 4.79 mg/g DW and range of 3.44 - 5.83 mg/g DW. Quercetin-3-*O*--*O*-galactoside had a seasonal average of 4.58 mg/g DW and range of 3.51 - 5.96 mg/g DW. The quantities of all three of these compounds were at maxima in September, 2006, when the leaves of *R. tomentosum* turn red.
Table 2.2  Environmental conditions\(^a\), percent yield\(^b\), and phenological stage of *R. tomentosum* seasonal collections.

<table>
<thead>
<tr>
<th>Date</th>
<th>Mean Temperature (Low-High) (°C)</th>
<th>Precipitation (mm)</th>
<th>Daylight (h)</th>
<th>Percent yield (w/DW)</th>
<th>Phenological stage</th>
</tr>
</thead>
<tbody>
<tr>
<td>June 13</td>
<td>3.2 (0.2-6.1)</td>
<td>1.3</td>
<td>20.3</td>
<td>21.3 ± 2.3</td>
<td>Leaf buds</td>
</tr>
<tr>
<td>June 27</td>
<td>4.7 (1.8-7.6)</td>
<td>2.0</td>
<td>20.7</td>
<td>24.0 ± 1.7</td>
<td>Leaf growth</td>
</tr>
<tr>
<td>July 13</td>
<td>10.3 (5.6-15.0)</td>
<td>0.8</td>
<td>20.1</td>
<td>23.5 ± 2.0</td>
<td>Flowering</td>
</tr>
<tr>
<td>July 26</td>
<td>9.3 (5.2-13.3)</td>
<td>0.8</td>
<td>19.0</td>
<td>23.0 ± 0.6</td>
<td>Seed pod development</td>
</tr>
<tr>
<td>August 9</td>
<td>7.2 (4.2-10.2)</td>
<td>0.3</td>
<td>17.6</td>
<td>23.7 ± 1.8</td>
<td>Seed pod maturation</td>
</tr>
<tr>
<td>August 23</td>
<td>9.0 (5.0-12.9)</td>
<td>0.7</td>
<td>16.1</td>
<td>22.7 ± 2.0</td>
<td>Leaf growth</td>
</tr>
<tr>
<td>Sept 8</td>
<td>6.7 (3.3-10.2)</td>
<td>1.5</td>
<td>14.4</td>
<td>25.0 ± 2.5</td>
<td>Leaf growth</td>
</tr>
<tr>
<td>Sept 30</td>
<td>3.0 (0.9-5.1)</td>
<td>1.2</td>
<td>12.1</td>
<td>25.3 ± 2.9</td>
<td>Leaf death- red</td>
</tr>
</tbody>
</table>

\(^a\) Mean temperature, precipitation and daylight hours are presented as the 14 day average preceding the 2006 collection date.

\(^b\) Extract percent yield was calculated as \(\frac{\text{extract weight}}{\text{dry plant material weight (g/g)}} \times 100\)
Figure 2.1 Chemical structures of the fifteen phenolic constituents analysed in *R. tomentosum* leaf extract.
Figure 2.2 Sample HPLC chromatograms of *R. tomentosum* leaf extract with detection at A) 325 nm and B) 280 nm.
Figure 2.3 Seasonal variation of the mean quantities ± SEM of the three most abundant compounds in *R. tomentosum* leaf extract; A) (+)-catechin, B) quercetin-pentoside, and C) quercetin-3-O--O-galactoside. Different letters indicate significant differences between collection dates at *p* < 0.05.
Similarly, procyanidin-B3 and procyanidin-B2, the fourth and fifth most abundant compounds, displayed seasonal maxima in September (Fig. 2.4). The seasonal average quantities of procyanidin-B2 and procyanidin-B3 were 2.93 mg/g DW and 3.91 mg/g DW, respectively. Procyanidin-B1 was one of the least abundant compounds with a seasonal average quantity of 0.99 mg/g DW and a maximal quantity in June, 2006 (Fig. 2.4).

In contrast, the quantities of the three caffeic acid derivatives did not follow this seasonal trend with maxima occurring in September (Fig. 2.5). Interestingly, it appears that caffeic acid derivative 1 and caffeic acid derivative 2 follow an opposite mirror image seasonal trend with their respective seasonal minima and maxima occurring in August. Caffeic acid derivative 1, the seventh most abundant compound, had a seasonal average quantity of 2.01 mg/g DW, with a seasonal maxima of 2.66 mg/g DW occurring in June, 2006. Caffeic acid derivative 2, the sixth most abundant compound, had a seasonal average of 2.62 mg/g DW, with a seasonal maxima of 3.68 mg/g DW occurring in July, 2006. Caffeic acid derivative 3, one of the least abundant compounds, had a seasonal average quantity of 0.58 mg/g DW, with a seasonal maxima of 1.07 mg/g DW occurring in August, 2006.

Myricetin, quercetin, quercetin-3-O-glucoside, and quercetin-3-O-rhamnoside had relatively low seasonal average concentrations (Fig. 2.6). Quercetin-3- O -rhamnoside showed a 500% spike in concentration in mid-July, which corresponded to R. tomentosum flowering. Quercetin and quercetin-3- O -glucoside both had seasonal minima at the end of August and maxima in September. Chlorogenic acid and p-coumaric acid also had relatively low seasonal
Figure 2.4 Seasonal variation of the mean quantities ± SEM of the three procyanidins compounds assessed in *R. tomentosum* leaf extract; A) procyanidin-B1, B) procyanidin-B2, and C) procyanidin-B3. Different letters indicate significant differences between collection dates at *p* < 0.05.
Figure 2.5 Seasonal variation of the mean quantities ± SEM of the three caffeic acid derivatives assessed in *R. tomentosum* leaf extract; A) caffeic acid derivative 1, B) caffeic acid derivative 2, and C) caffeic acid derivative 3. Different letters indicate significant differences between collection dates at \( p < 0.05 \).
Figure 2.6  Seasonal variation of the mean quantities ± SEM of four quercetin derivatives assessed in *R.tomentosum* leaf extract; A) myricetin, B) quercetin, C) quercetin-3-*O*-rhamnoside, and D) quercetin-3-*O*-glucoside. Different letters indicate significant differences between collection dates at $p < 0.05$. 
average concentrations but they displayed similar seasonal trends with a maximal concentration at the end of June, which corresponded to the period with the longest sunlight hours (Fig. 2.7).

The seasonal trend was also assessed for the total sum of the fifteen phenolic compounds included in this study (Fig. 2.8). It is interesting to note that each of the fifteen phenolic compounds displayed a significant seasonal variation in quantity however the sum total did not. The variable nature of the seasonal quantities of each phenolic constituent resulted in a high standard error for their sum total, which may have prevented the detection of a significant trend. Although there was no statistically significant seasonal variation, there was a clear growth dilution trend of total phenolics. At the beginning of the growing season soon after the snow cover melts there was a seasonal minimum concentration of total compounds, namely 28.8 mg/g DW. Only 2 weeks later this concentration rose to 38.6 mg/g DW, which corresponded to the timing of *R. tomentosum* leaf bud development. The total concentration then falls throughout July as the leaves grow, and by the end of July begins to increase in concentration again to seasonal maxima in September, at 40.0 mg/g DW, which corresponded to the timing of *R. tomentosum* leaves turning red.

### 2.3.3 Seasonal variation of medicinal properties.

As expected, the DPPH antioxidant activity of *R. tomentosum* displayed a similar seasonal trend as the total sum of compounds, whereas there was no such relationship with the TNF-α anti-inflammatory activity (Fig. 2.9). Accordingly, there was a significant correlation between antioxidant activity DPPH IC$_{50}$ and the total sum of constituents.
Figure 2.7  Seasonal variation of the mean quantities ± SEM of two acid compounds assessed in *R.tomentosum* leaf extract; A) chlorogenic acid, and B) p-coumaric acid. Different letters indicate significant differences between collection dates at \( p < 0.05 \).
**Figure 2.8** Seasonal variation of the total quantity ± SEM of all fifteen compounds assessed in *R. tomentosum* leaf extract.
Figure 2.9 Seasonal variation of the pharmacological activities ± SEM assessed in *R.tomentosum* leaf extract; A) antioxidant DPPH activity (IC₅₀ µg/ml) and, B) TNF-Alpha response in stimulated monocytes (% TNF-alpha concentration relative to vehicle control). Different letters indicate significant differences between collection dates at *p* < 0.05.
(r = -0.774; p = 0.024), but not between anti-inflammatory activity and total sum of constituents. The average DPPH antioxidant activity was 103.4 µg/ml. Antioxidant activity was low at the beginning of the season and the seasonal minima, 113.9 µg/ml, occurred in July when the plant produced flowers. After which, the activity consistently increased to the seasonal maxima, 89.6 µg/ml, at the beginning of September when the leaves were turning red (Fig. 2.9A). The positive control, L-ascorbic acid, had an IC₅₀ of 46.6 µg/ml, resulting in an average R. tomentosum extract percentage of positive control of 45%. The positive control ratio (ascorbic acid logIC₅₀/plant extract logIC₅₀) of our extract was 0.83.

The average TNF-α anti-inflammatory activity was 64.3% of vehicle control, with the minima occurring in July (98.0%) and maxima in August (41.1%), after which time the activity proceeded to decrease throughout September when the leaves of R. tomentosum are turning red (Fig. 2.9B). Antioxidant activity was not correlated to anti-inflammatory activity (r = 0.62; p = 0.10).

2.3.4 Relationship between medicinal properties, phytochemicals and environmental conditions.

By conducting partial correlations of each phenolic constituent and the DPPH antioxidant or TNF-α anti-inflammatory activities, we were able to identify the most active compounds associated with each medicinal property. Antioxidant activity (1/IC₅₀) had a significant positive correlation with (+)-catechin (r = 0.80; p = 0.02) and procyanidin-B2 (r = 0.83; p = 0.01), the first and fifth most abundant compounds identified respectively. Anti-
Figure 2.10 Correlation between daily sunlight hours and A) antioxidant DPPH activity (1/IC50 µg/ml) and, B) anti-inflammatory TNF-alpha activity (1/% TNF-alpha concentration relative to vehicle control).
inflammatory activity (1/[% TNF-α response relative to the vehicle control]) had a significant negative correlation with caffeic acid derivative 1 (r = -0.72; p = 0.04), the seventh most abundant compound.

Antioxidant and anti-inflammatory activities of *R. tomentosum* leaves were also assessed for their relationship to the environmental conditions, including precipitation, temperatures, and photoperiod sunlight hours. Surprisingly, daily photoperiod was negatively correlated to antioxidant activity (1/DPPH IC\textsubscript{50}) (r = 0.72; p = 0.05), and anti-inflammatory activity 1/(% TNF-α response relative to the vehicle control) (r = 0.82; p = 0.01) (Fig. 2.10). By conducting partial correlations of each phenolic constituent and environmental condition we were able to identify the most active compounds responsible for this trend. Daily sunlight was significantly negatively correlated to the quantity of (+)-catechin (r = -0.73; p = 0.04), procyanidin-B2 (r = -0.71; p = 0.05), procyanidin-B3 (r = -0.73; p = 0.04), and the total sum of compounds (r = -0.70; p = 0.05).

### 2.4 Discussion

Our results demonstrate the seasonal variation of the phenolic constituents of *R. tomentosum* leaf ethanol extract, in parallel with its medicinal activities and the environmental growing conditions. The plant samples were collected from Iqaluit, Nunavut, which is situated in the Low Arctic and characterized by a short summer season, long daylight hours in the summer, and low rain fall (Bliss, 2000). Plants thriving in Arctic tundra have particular genetic, physiological, and phytochemical adaptations for growth in this
climate (Bliss, 2000; Chapin & Shaver, 1985). Medicinal plants, such as *R. tomentosum*, are selected by their users for their medicinal properties, which in turn are largely determined by the plant’s phytochemical composition. Although it is widely accepted that plants’ phytochemical composition varies according to their developmental stage, environmental conditions, and the seasonal timing, very little is known about plants growing in the Arctic ecosystem.

The major phenolics present in the *R. tomentosum* leaves characterized in this study were (+)-catechin, quercetin-pentoside, and quercetin-3--O-galactoside, which is in agreement with previous characterization studies (Saleem et al., 2010). In our study, the quantities of all fifteen phenolic constituents we quantified significantly varied throughout the season. The sum total phenolic content had no statistically significant seasonal variation, however it displayed a typical trend of growth dilution. At this study site, the snow melted in late May when the tundra plants were exposed to sunlight and above freezing temperatures for the first time in 8 months (Environment Canada, 2010). Soon afterwards, *R. tomentosum* unfolded its leaf buds and activated its metabolism for the short upcoming summer, resulting in an accumulation of phenolics by the end of June. The warmest yearly temperatures occur in July, when the leaves rapidly grow and flowers are produced, which caused the dilution of phenolics and allocation of resources away from the leaves and into reproductive organs. After flowering, the phenolic content consistently increased again until it reached its seasonal maxima in September, also the timing of the maximal antioxidant activity. The fall (late-August to September at this latitude) is
traditionally known to be the optimal time for harvest of this medicinal plant (Black et al., 2008; Ziegler et al., 2009; Mallory et al., 2004).

The antioxidant and anti-inflammatory properties of *R. tomentosum* may be a factor in the selection of this plant as a medicine by multiple Aboriginal peoples of Canada. Phenolics are well known antioxidant compounds, and many of the detected phenolic constituents are commercially available supplements with health benefits established in clinical trial studies (Moore et al., 2009). In our study, the antioxidant activity found in *R. tomentosum* leaf extract was similar to previously reported values (Fraser et al., 2007; Harbilas et al., 2009) despite varying sampling locations, seasonal collection dates, species, or testing procedures. Peak antioxidant activity corresponded with peak phenolic content, both occurring in September.

The use of phenolics as anti-inflammatory agents is well characterized. Inflammatory signaling cascades include free radicals, which lead to the activation of nuclear factor κB and the transcription of TNF-α inflammatory cytokine (Winrow et al., 1993). Many traditionally used medicinal plants and phytochemical compounds, such as parthenolide, are known to target these pathways as anti-inflammatory therapies (D'Acquisto et al., 2002). The peak anti-inflammatory activity was observed at the end of August 2006, approximately 2 weeks prior to the peak antioxidant activity, and the traditional collection period for this medicinal plant (Black et al., 2008; Ziegler et al., 2009; Mallory et al., 2004). Methanol extracts of Labrador Tea (*R. groenlandicum*) have also been previously reported to possess anti-inflammatory activity in a rat paw edema model (Dufour et al., 2007).
The antioxidant activity \((1/DPPH \text{IC}_{50})\) was correlated to the quantity of the total phenolics, a trend that has been well established (Surveswaran et al., 2010; Mustafa et al., 2010). Of the specific constituents, antioxidant activity was positively correlated to the quantity of (+)-catechin and procyanidin-B2, suggesting their dominant role as plant photoprotectant antioxidants. Previous studies have highlighted the importance of photoprotection particularly in environments with low temperatures and a long photoperiod (Dixon and Paiva, 1995; Grace, 2005). Surprisingly however, (+)-catechin and procyanidin-B2 were negatively correlated to total daylight hours. In temperate climates and greenhouse conditions, it is well established that plants increase their phenolic content in response to elevated solar radiation exposure (Jaakola et al., 2004; Stark et al., 2008; Tegelberg et al., 2002). In our study, the pronounced growth dilution trend may have hidden any such correlation since the timing of the highest sun exposure (June) also corresponds to rapid leaf growth of *R. tomentosum*.

A correlation between anti-inflammatory activity and phenolic content has been previously suggested (Fawole et al., 2010), however this was not the case in our study. As seen with the antioxidant activity, anti-inflammatory activity was negatively correlated with daylight hours. Surprisingly, there was a negative correlation between anti-inflammatory activity and caffeic acid derivative 1, which was only the 7th most abundant characterized phytochemical. Caffeic acid phenethyl ester has demonstrated anti-inflammatory activity *in vivo* (Frenkel et al., 1993), although each caffeic acid derivative may have varying activities. In fact, many of the compounds quantified in this study have been shown to possess *in vitro*
and *in vivo* anti-inflammatory activity, including catechin, chlorogenic acid, procyanidins, and quercetin derivatives (Matsuoka et al., 1995). As none of these compounds were significantly correlated to the anti-inflammatory activity, it is hypothesized that individually none of them were present in large enough amounts to influence the anti-inflammatory activity of *R. tomentosum*.

### 2.5 Conclusion

Considering the wide-spread use of *R. tomentosum* as a medicinal plant to First Nations and Inuit peoples of Canada, it was important to characterize its phenolic constituents and medicinal activities, as well as to determine how these properties vary throughout the growing season. Medicinal plants were an important source of nutrients and antioxidants, particularly for the Inuit who traditionally relied largely on a meat based diet. The seasonal variation of phenolic constituents and medicinal properties has implications for optimal harvest time and consistency of product. Our results show a significant seasonal variation of phenolic constituents and medicinal properties of *R. tomentosum*, and corroborate traditional knowledge of optimal harvest time at the end of August to September for this location.
CHAPTER THREE

Modulation of the effects of methylmercury on rat neurodevelopment and behaviour by co-exposure with Northern Labrador Tea (Rhododendron tomentosum spp. subarcticum).

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1. Contributed to original ideas, performed animal exposure, early development measures, and behavioral tests, assessed NMDA receptor binding, tested GSH, TBARS, EROD, BROD, PROD levels in animal samples, data analysis, and manuscript preparation. Centre for Advanced Research in Environmental Genomics (CAREG), University of Ottawa. Hazard Identification Division, Environmental Health Science and Research Bureau, Health Canada.


3. Measured total mercury tissue concentrations (B.Sc honours student). Centre for Advanced Research in Environmental Genomics (CAREG), University of Ottawa.

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6. Provided laboratory resources and expertise in rat behavioral tests, manuscript revisions. Hazard Identification Division, Environmental Health Science and Research Bureau, Health Canada.

7. Provided laboratory resources and expertise in NMDA receptor binding, manuscript revisions. Community Health Sciences Program, University of Northern British Columbia.

8. Provided laboratory resources, expertise and manuscript revisions for plant extraction. Centre for Advanced Research in Environmental Genomics (CAREG), University of Ottawa.

9. Contributed to original ideas, performed animal necropsy, and revised the manuscript. Hazard Identification Division, Environmental Health Science and Research Bureau, Health Canada.
3.1 Introduction

Canadian Inuit are exposed to a variety of persistent organic pollutants and heavy metals, most notably methylmercury (MeHg), through the consumption of their traditional diet (Van Oostdam et al., 2005). Epidemiological studies conducted with seafood-consuming populations in the Faroe Islands and New Zealand have reported adverse health effects associated with prenatal MeHg exposure (Debes et al., 2006; Crump et al., 1998). In contrast, studies conducted in the Seychelles Islands and in the Canadian Arctic did not find such association (Despres et al., 2005; Davidson et al., 2006). These discrepancies may be explained by differing factors such as genetic susceptibilities, study protocols, co-exposure with other environmental toxicants, and maternal nutritional status (Jacobson, 2001).

There is a growing body of literature on the effects of dietary nutrients on MeHg toxicity. Most of these studies focused on compounds commonly found in MeHg-contaminated food sources or which may potentially interfere with MeHg molecular mechanisms of toxicity (selenium, fatty acids, thiol-containing compounds and vitamins). Divergent findings were often reported, but globally these studies suggest that nutrients can modulate the adverse outcomes resulting from exposure to MeHg (Chapman and Chan, 2000; Rice, 2008).

Among the best characterized mechanisms of MeHg neurotoxicity are excitotoxicity and oxidative stress (Yin et al., 2007; Aschner et al., 2007). Perturbation of glutamate transport in astrocytes by MeHg can lead to over-stimulation of the N-methyl-D-aspartate receptors (NMDA-R), causing excitotoxicity (Yin et al., 2007). Excitotoxicity is characterized
by Ca\textsuperscript{2+} imbalance, nitrotyrosine release, oxidative stress and apoptosis (Michaelis, 1998). The NMDA-R is part of the glutaminergic system which is crucial for neuronal plasticity, learning and memory (Scheetz and Constantine-Paton, 1994). NMDA-R perturbation is associated with schizophrenia (Gaspar et al., 2009), Huntington’s disease (Levine et al., 2010), Alzheimer’s and Parkinson’s disease (Koutsilieri and Riederer, 2007). During fetal development, NMDA-Rs are also involved in the establishment of neuronal circuitry (Haberny et al., 2002), which might constitute an additional target of MeHg toxicity. While NMDA-R mediated excitotoxicity can lead to the generation of reactive oxygen species (Gasso et al., 2001), MeHg can also generate oxidative stress through direct perturbation of mitochondrial functions (Aschner et al., 2007). The resultant oxidative stress can lead to cell membrane damage, calcium deregulation, enzyme and cell signalling interference, microtubule disassembly, and ultimately, cell death (do Nascimento et al., 2008).

MeHg co-exposure studies provide further evidence on the role of glutamate excitotoxicity and oxidative stress in MeHg-induced neurotoxicity. MeHg-induced neurotoxicity can be mitigated by co-exposure with NMDA-R antagonists (Zhang et al., 2003; Faro et al., 2002). Various purified phytochemicals or plant extracts have been shown to confer some protection against NMDA receptor agonist-induced excitotoxicity and oxidative stress in vivo and in vitro (Silva et al., 2008; Campos-Esparza et al., 2009; Chen et al., 2008; Cho and Lee 2004) and interestingly, similar findings have been reported following co-exposure with MeHg (Lucena et al., 2007; Yongjin et al., 2008; Farina et al., 2005). Possible mechanisms involved in the mitigation of MeHg effects by phytochemicals may
include the reduction of reactive oxygen species, activation of enzymatic antioxidant systems, restoration of the mitochondrial membrane potential, and modulation of cell signalling pathways (Campos-Esparza et al., 2009). Dietary phytochemicals may also affect additional endpoints such as MeHg bioavailability and pharmacokinetics (Passos et al., 2007; Laird et al., 2009).

The low consumption of fruits and vegetables in Canadian Arctic communities, which may be explained by geographical, economical and cultural reasons (Van Oostdam et al., 2005), can limit plant-based antioxidant intake in these populations. *Rhododendron tomentosum* ssp. *subarcticum*, commonly known as Northern Labrador Tea, is an abundant woody tundra plant commonly used by Canadian Inuit as a traditional medicinal tea for the prevention and treatment of respiratory illnesses (Black et al., 2008). Ethanolic extracts of *R. tomentosum*, hereafter referred to as “Tea”, have high free radical scavenging activity in vitro and contain many well characterized antioxidant phytochemicals such as quercetin and caffeic acid (Black et al., 2011, submitted). Consumption of this plant, which is already part of the cultural background of many northern communities, may therefore represent a feasible method to increase phytochemical-based antioxidant intake in Canadian Arctic populations exposed to MeHg.

The current study was designed to test if co-exposure with antioxidants present in Tea can mitigate methylmercury-induced neurotoxicity in a rat perinatal exposure model. Dams were dosed daily throughout gestation and lactation with MeHg and Tea administered either individually or together. The effects of co-exposure on pup growth,
development, tissue mercury concentrations and oxidative status, neuromuscular functions, neurobehavior and brain NMDA receptor levels were assessed.

3.2 Materials and Methods

3.2.1 Plant extract preparation

Please refer to section 2.2.1. *Rhododendron tomentosum* ssp. *subarcticum* plant material used for this study was collected in a single batch in July 2006 near Iqaluit, Nunavut.

3.2.2 Tea treatment animal pilot project

All experimental procedures adhered to the Canadian Council on Animal Care Guidelines and were approved in advance by Health Canada’s Animal Care Committee. Adult (11 weeks) female Sprague-Dawley rats were obtained from Charles River (St-Constant, QC, Canada) and singly housed in 35 x 30 x 17 cm hanging polycarbonate cages on shaved wood beddings with *ad libitum* access to tap water and rat chow. The room was maintained at 22 ± 1°C and 50 ± 10% humidity on a reversed 12-hour light cycle (dark phase from 7:00 to 19:00).

After a 1-week acclimatization period, rats were randomly assigned to a treatment group; Tea (100 mg Tea extract/KgBW/d, n = 10), or Control (vehicle, n = 10). Rats were dosed daily for 1-week. *R. tomentosum* extract dissolved in high-grade 100% ethanol (500 mg/ml) was applied to small graham cookies (Teddy Graham, Nabisco Ltd., Toronto,
Canada) and the ethanol was allowed to evaporate overnight in a fume hood. Rats were monitored on a daily basis for body weight, morbidity, and water and food consumption by trained veterinary technicians for 2-weeks. At the termination of the study, rats were sacrificed under isoflurane anaesthesia. Since no signs of toxicity were observed no samples were collected and we proceeded to our main study.

3.2.3 Animal treatment and tissue collection

All experimental procedures adhered to the Canadian Council on Animal Care Guidelines and were approved in advance by Health Canada’s Animal Care Committee. Adult (11 weeks) male and female Sprague-Dawley rats were obtained from Charles River (St-Constant, QC, Canada) and kept in 35 x 30 x 17 cm hanging polycarbonate cages on shaved wood beddings with ad libitum access to tap water and rat chow. The room was maintained at 22 ± 1°C and 50 ± 10% humidity on a reversed 12-hour light cycle (dark phase from 7:00 to 19:00).

After a 2-week acclimatization period, breeding was initiated by introducing two females into the male’s home cage. Females were checked daily for the presence of a vaginal plug or sperm, which was defined as gestational day 0 (GD 0). At GD 0 females were housed individually and randomly assigned to one of the four treatment groups; MeHg (2.0 mg MeHg/KgBW/d, n = 12), Tea (100 mg Tea extract/KgBW/d, n = 10), MeHg + Tea (2.0 mg MeHg/KgBW/d & 100 mg Tea extract/KgBW/d, n = 11), or Control (vehicle, n = 10). Dams were weighed and dosed daily from GD 1 to PND 21. To best approximate human MeHg
scenarios, animal dosing was conducted using a spiked dietary exposure method. *R. tomentosum* extract dissolved in high-grade 100% ethanol (500 mg/ml) was applied to small graham cookies (Teddy Graham, Nabisco Ltd., Toronto, Canada) and the ethanol was allowed to evaporate overnight in a fume hood. MeHg dissolved in corn oil (2 mg/ml) was then applied to the cookie. Control and Tea-treated dams were dosed with a cookie containing the same weight-adjusted volume of corn oil. Offspring were weighed and counted at parturition, and litters were culled to four males and four females on PND 4. Dams and pups (grouped by gender) were weighed daily, and morbidity monitored by veterinary technicians. At PND 14 and PND 21 one male and one female from each litter were sacrificed under isoflurane anaesthesia. In addition, at PND 14 and PND 21 one male and one female from each litter were sacrificed by decapitation for the collection of gene transcriptional analyses samples. Brains were cut along the longitudinal axis and half brains were kept for the analysis of Hg residues, oxidative stress and NMDA-R. At PND 21 the dams were also sacrificed using the same procedure. Blood, brain and liver tissues were collected, flash frozen in liquid nitrogen and stored at -80°C.

**3.2.4 Pup early development measures**

Eye and ear opening were assessed on a daily basis in all pups, beginning on PND 11. Grip strength was assessed in the same subset of pups at both PND 12 and 15 using the previously described forepaw suspension test (Bowers et al., 2004). Briefly, each pup was suspended by placing its forepaws on a horizontal 3 mm radius metal wire, 30 cm above soft bedding. The time the pup held onto the wire was recorded for a maximum of 60
seconds. Testing was completed during the first 4 hours of the pup’s active dark cycle, and in a staggered treatment group order to ensure equal representation in each time block of testing.

3.2.5 Pup behaviour assessment

Behavioural tests were performed on a subset of one male and one female pup from each litter during the first 4 hours of their active dark cycle under 20-watt red bulb lighting. Tests were conducted in a sound attenuated room with background white noise generated by 2 speakers (80 dB at source) located in opposite corners of the room.

The open field test was conducted on PND 16 pups to determine spontaneous motor activity and exploratory behaviour (Russell, 1973). Animals were tested individually by placing the pup in the start corner of a clean 43 x 43 x 30 cm polypropylene chamber. Chambers were equipped with infrared beams and detectors dividing the floor area into 289 2.5 x 2.5 cm squares (model ENV-515, Med Associates Inc., USA). The location of the pup was monitored for 25 minutes by recording photocell beam breaks, using Activity Monitor Software 4.33 (Med Associates Inc. USA) on a computer located in an adjacent room. Ambulatory time was defined as the time the pup spent crossing the squares and vertical counts as the number of vertical rearings detected by a separate set of infrared beams located 3.75 cm above the chamber’s floor. Thigmotaxis (preference for the periphery) was estimated by the amount of time spent within 5 cm of the chamber walls.
The acoustic startle test was conducted on PND 20 pups to evaluate sensorimotor gating and acoustic reactivity (Geyer and Swerdlow, 2001). Automated startle chambers (model 6500-0091-E, San Diego Instruments Inc., USA) with a ventilated clear holding tube mounted on a motion detector and a wideband audio speaker were used. The pup was allowed to acclimatize for 5 minutes with 65 dB of background white noise. Startle responses of each pup were measured over 120 trials consisting of a prepulse stimulus (0, 70, 85, 100 dB) followed 50 ms later with a 50 ms startle stimulus (105, 112, 118 dB). Individual trials were spaced by random intervals ranging from 5 to 10 seconds. The first and last 12 trials consisted of startle only trials (prepulse value = 0 dB) with 3 trials at each startle intensity (105, 112, and 118 dB). The remaining trials consisted of prepulse trials with 8 trials at each startle-prepulse combination (4 prepulse and 3 startle intensities). In addition, 10 null trials with no acoustic stimuli were randomly presented throughout the testing period. Data was recorded on a computer in an adjacent room (SR-LAB Startle Reflex System 5.0, San Diego Instruments Inc. USA). Startle response time and amplitude were averaged over all trials for each startle-prepulse combination. Amplitude was normalized to pup weight by dividing the amplitude by the weight of the pup the day of the trial. The prepulse inhibition (PPI), or the suppression of the startle response by a prepulse, was calculated as \( \% \text{ PPI} = \left[ 1 - \left( \frac{\text{startle amplitude for pulse + prepulse trial}}{\text{startle amplitude for pulse only trial}} \right) \right] \times 100 \).
3.2.6 Mercury quantification

Total mercury (THg) was quantified by thermal decomposition followed by dual step gold amalgamation and detection by cold vapour atomic absorption spectrophotometry using a SP-3D mercury analyzer (Nippon Instruments, Japan) as described previously (Basu et al., 2005; Al-Reasi et al., 2007). Approximately 10 mg of unprocessed sample was placed in a ceramic boat covered with layers of additive M (sodium carbonate and calcium hydroxide) and additive B (aluminum oxide), and placed into the thermal decomposition chamber for analysis.

MeHg concentration was assessed as described previously (Cai et al., 1997; Al-Reasi et al., 2007). Briefly, the samples were subject to alkaline digestion followed by acid leaching. Organomercuric species were extracted in dichloromethane, filtered and placed in amber vials for analysis by capillary gas chromatography (HP6890 Series, Agilent, USA) coupled with atomic fluorescence spectrometry (PSA Merlin Millennium Detector, PS Analytical, Orpington, UK).

Quality control of THg and MeHg recovery was assessed every 5 samples with standard reference material (DORM-2, dogfish muscle, National Research Council, Ottawa, Canada), which remained within the certified values of 4.64 ± 0.26 mg/kg and of 4.47 ± 0.32 mg/kg for THg and MeHg respectively. The detection limit for THg and MeHg was 0.01 ng and 0.02 ng Hg, respectively.
3.2.7 NMDA receptor binding

NMDA receptor binding assay was performed as previously described (Basu et al., 2007). Cellular membrane protein extracts were prepared from whole brain tissue from pups and dams as described elsewhere (Basu et al., 2006). 30 µg of prepared protein extracts were re-suspended in Binding Buffer (50 mM Tris pH 7.4, 100 µM glycine, 100 µM L-glutamate) and incubated with 5 nM [³H]-MK801 (Cat # NET972250UC, Lot 3557396, 22 Ci/mmoll, PerkinElmer, Boston, MA, USA) in MultiScreen_HST plates with 1.0 µm glass fibre filter (Millipore, Boston, MA, USA) for 60 minutes with gentle agitation at room temperature. The samples were vacuum-transferred to glass filters and rinsed three times with Binding Buffer. The filters were dried under a heat lamp for 60 minutes and 25 µl of Liquid Scintillation Cocktail (Ultima Gold, Perkin Elmer) was added. Samples were read immediately on a Chameleon Multi-technology Plate Reader 425-106 (Hidex Oy, Turku, Finland) with a counting efficiency of 15%. Specific binding was determined as the difference between the binding of [³H]-MK801 on samples pre-incubated or not with unlabelled MK801. Inter and intra-plate variations were 8% and 6%, respectively.

3.2.8 Biochemical analyses

Brain and liver tissues were homogenized with 1.5 volumes ice cold 0.05 M Tris 0.15% KCl buffer, pH 7.4. Reduced glutathione (GSH) was measured in brain homogenates and serum using a colorimetric kit (Calbiochem, San Diego, CA, USA). Lipid peroxidation was assessed in brain homogenates and serum by thiobarbituric acid reactive substances (TBARS) using a fluorescence method described previously (Yagi, 1982). Liver homogenates
were centrifuged for 20 minutes at 9,000 x g. The supernatant was assayed for benzyloxyresorufin-O-dealkylase (BROD), ethoxyresorufin-O-deethylase (EROD) and pentoxyresorufin-O-dealkylase (PROD) activities as described previously (Burke et al., 1985).

### 3.2.9 Statistical analysis

Datasets were assessed for normality with the Shapiro-Wilks test and for homogeneity of variance with Levene’s test. Grip strength data was log transformed and analyzed with repeated measures analysis of variance. Statistical analyses were performed as appropriate by one-way or two-way ANOVA followed by Tukey’s post-hoc test. When data was available for more than one pup per litter, a nested ANOVA was used. Non-parametric data was analyzed with the Kruskal-Wallis test followed by pairwise Mann-Whitney U tests when appropriate. Analysis was performed with SPSS version 15.0, 2006 (SPSS Inc., Chicago, IL, USA). Differences were considered statistically significant when $p < 0.05$.

### 3.3 Results

#### 3.3.1 Growth and development

Dam weight gains during gestation were not affected by any treatment (Fig. 3.1A). After parturition, dam weights were lower in the MeHg and MeHg + Tea treatment groups but these differences were not statistically significant. Similar pronounced growth impairments were observed for pups of the MeHg and MeHg + Tea treatment groups in both genders (Fig. 3.1B). There were no differences in litter size and pup gender ratio across
Figure 3.1  Body weight ± SEM of A) dams (control n = 10, Tea n = 10, MeHg n = 8, MeHg + Tea n = 8), and B) pups (control n = 10, Tea n = 10, MeHg n = 6, MeHg + Tea n = 6). Pup weight was determined as the average weight of each pup per litter. Different letters indicate significant treatment group differences at $p < 0.05$. 

Figure 3.1
Table 3.1 Pup litter size and gender ratio ± SEM (males/total).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Litter Size</th>
<th>Gender Ratio (males/total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>16.6 ± 0.5</td>
<td>0.53 ± 0.04</td>
</tr>
<tr>
<td>Tea</td>
<td>15.6 ± 0.5</td>
<td>0.51 ± 0.03</td>
</tr>
<tr>
<td>MeHg</td>
<td>15.3 ± 0.6</td>
<td>0.57 ± 0.07</td>
</tr>
<tr>
<td>MeHg + Tea</td>
<td>16.2 ± 0.9</td>
<td>0.47 ± 0.04</td>
</tr>
</tbody>
</table>
Table 3.2 Mean percent mortality ± SEM (range) of pups within PND1-4 and PND 5-21, and the number of litters affected. Different letters indicate significant treatment group differences at $p \leq 0.05$.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>PND 1 – 4</th>
<th></th>
<th>PND 5 - 21</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percent mortality</td>
<td>Litters Affected</td>
<td>Percent mortality</td>
<td>Litters Affected</td>
</tr>
<tr>
<td>Control</td>
<td>0.6% (0-5.6%)$^a$</td>
<td>1/10.</td>
<td>0.0%$^a$</td>
<td>0/10.</td>
</tr>
<tr>
<td>Tea</td>
<td>2.5% (0-7.1%)$^a$</td>
<td>3/10.</td>
<td>0.0%$^a$</td>
<td>0/10.</td>
</tr>
<tr>
<td>MeHg</td>
<td>51.3% (0-100%)$^b$</td>
<td>11/12.</td>
<td>45.3% (0-100%)$^b$</td>
<td>5/8.</td>
</tr>
<tr>
<td>MeHg + Tea</td>
<td>45.1% (0-100%)$^b$</td>
<td>10/11.</td>
<td>29.8% (0-100%)$^b$</td>
<td>5/8.</td>
</tr>
</tbody>
</table>
treatment groups (Table 3.1). Exposure to MeHg administered either alone or with Tea resulted in high pup mortality rates (Table 3.2). Mortality rates observed before PND 4 culling were similar to those reported for the same MeHg exposure protocol (Pelletier et al., 2009). Deaths after PND 4 were mainly concentrated in 2 litters in each of the MeHg ($n = 8$) and MeHg + Tea ($n = 8$) treatment groups, which accounted for more than half of the mortality rates. Although MeHg and Tea administered separately did not significantly affect the timing of pups’ eye opening, their co-administration resulted in precocious eye opening in both genders (Fig. 3.2). There was no treatment group effect on the age of ear opening (data not shown).

As expected, grip strength increased from PND 12 to PND 15 in all pups. Overall, male pups treated with MeHg and MeHg + Tea exhibited significantly poorer performances on the grip strength test than control males (Fig. 3.3A). A similar trend was observed in female pups, but the effect was not statistically significant (Fig. 3.3B).

### 3.3.2 Behavioural assessment

Pups from the MeHg and MeHg + Tea treatment groups displayed significant hyperactivity in open field testing, as indicated by an approximately 3-fold increase in ambulatory time compared to control pups (Fig. 3.4A). The same trend was observed for ambulatory distance and counts (data not shown). This hyperactivity was also accompanied by a significant loss of thigmotaxis in both treatment groups (Fig. 3.4B), while the number of vertical rearings remained similar in all treatment groups (data not shown).
Figure 3.2 Percentage of pups per litter with both eyes open by postnatal day age (control $n = 10$, Tea $n = 10$, MeHg $n = 6$, MeHg + Tea $n = 7$). Different letters indicate significant treatment group differences at $p < 0.05$. 
Figure 3.3  Suspension time (log \(^{10}\) transformed) ± SEM in the grip strength test at PND 12 & 15 in A) male pups (control \(n = 10\), Tea \(n = 10\), MeHg \(n = 4\), MeHg + Tea \(n = 7\)) and B) female pups (control \(n = 10\), Tea \(n = 10\), MeHg \(n = 6\), MeHg + Tea \(n = 7\)). Different letters indicate significant treatment group differences at \(p < 0.05\).
Figure 3.4  Total time ± SEM spent A) in motion and B) in the center of the open field for male (control n = 10, Tea n = 10, MeHg n = 5, MeHg + Tea n = 5) and female (control n = 10, Tea n = 10, MeHg n = 6, MeHg + Tea n = 7) pups. Different letters indicate significant treatment group differences at p < 0.05.
Exposure to MeHg and MeHg + Tea significantly increased pup startle response time (Fig. 3.5). Pup’s acoustic startle response amplitude and prepulse inhibition was similar across all treatment groups (data not shown).

3.3.3 Tissue mercury residue

THg and MeHg concentrations in the blood and brain of male and female pups were similar and presented as litter averages as µg/g wet weight in brain tissues and mg/L in blood (Fig. 3.6). From PND 14 to PND 21, MeHg concentration declined by approximately 60% in the brain and 20% in the blood of MeHg and MeHg + Tea treated pups, due to low MeHg lactational transfer and dilution by growth (Stringari et al., 2008). Interestingly, pups exposed to MeHg + Tea presented higher blood and brain THg and MeHg concentrations than those exposed to MeHg alone, but this trend only reached statistical significance in blood at PND 21 (Fig. 3.6). Co-exposure may also have affected MeHg speciation. At PND 14, the un-metabolized MeHg accounted for the totality of the mercury measured in blood and brain of pups exposed to MeHg alone, while it represented about 84 ± 9% and 85 ± 8% of the total Hg measured in the blood and brain of pups exposed to MeHg + Tea, respectively. However, this trend only reached statistical significance in blood at PND 21 (Fig. 3.7).

3.3.4 NMDA receptor binding assay

At PND 14, none of the treatment groups significantly affected NMDA-R levels in pup brains (Fig. 3.8A). However, at PND 21, NMDA receptor levels in both male and female pups
Figure 3.5  Acoustic startle latency time ± SEM of pups following a 105, 112, or 118 dB startle stimuli (control $n = 10$, Tea $n = 10$, MeHg $n = 6$, MeHg + Tea $n = 6$). Averaged male and female littermate values are presented. Different letters indicate significant treatment group differences at $p < 0.05$. 
Figure 3.6  Total mercury (THg) and methylmercury (MeHg) ± SEM in A) brain tissue of PND 14 pups (µg/g) (MeHg n = 3, MeHg + Tea n = 5), B) brain tissue of PND 21 pups (µg/g) (MeHg n = 4, MeHg + Tea n = 6), C) blood of PND 14 pups (mg/L) (MeHg n = 2, MeHg + Tea n = 4), D) blood of PND 21 pups (mg/L) (MeHg n = 4, MeHg + Tea n = 6). Different letters indicate significant treatment group differences at $p < 0.05$. 

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Figure 3.7  Percent methylmercury ± SEM in pups’ A) brain tissue at PND 14 (MeHg $n = 3$, MeHg + Tea $n = 5$) and PND 21 (MeHg $n = 4$, MeHg + Tea $n = 6$), and B) blood at PND 14 (MeHg $n = 2$, MeHg + Tea $n = 4$) and PND 21 (MeHg $n = 4$, MeHg + Tea $n = 6$). Different letters indicate significant treatment group differences at $p < 0.05$. 
exposed to MeHg were almost twice as high as those measured in control pups. This effect was completely eliminated by co-exposure with Tea (Fig. 3.8B). There were no significant treatment effects on dam brain NMDA-R density (Fig. 3.9).

### 3.3.5 Oxidative stress

The effects of MeHg and Tea on lipid peroxidation, as assessed by TBARS, were tissue and developmental stage-specific. In serum, MeHg triggered a 4 to 6 fold increase in TBARS (Fig. 3.10A). Despite the observation that Tea had no effect on serum lipid peroxidation, its co-administration counteracted the MeHg effect at PND 21, with TBARS returning to control values. In the cerebrum, no effects were observed at PND 14, but at PND 21, exposure to Tea and MeHg + Tea resulted in significantly lower TBARS levels than those observed in control and MeHg treatment groups (Fig. 3.10B). In the cerebellum, MeHg and Tea administrated separately caused only modest, non-significant increases in TBARS at PND 14, but their co-exposure resulted in a significant 3-fold increase of lipid peroxidation. This exacerbation of MeHg effects by Tea appeared to be transitory as it was not observed at PND 21 (Fig. 3.10C). It is possible that brain region-specific levels of endogenous antioxidants were responsible for this variable response. None of the treatment groups had significantly altered GSH levels in serum, cerebellum or cerebrum tissues for male or female pups (data not shown).
Figure 3.8  NMDA receptor binding (fmol/mg protein) ± SEM in pup cerebrum at A) PND 14 males (control n = 5, Tea n = 5, MeHg n = 5, MeHg + Tea n = 5) and females (control n = 5, Tea n = 5, MeHg n = 3, MeHg + Tea n = 5), and B) PND 21 males (control n = 5, Tea n = 5, MeHg n = 3, MeHg + Tea n = 5) and females (control n = 5, Tea n = 5, MeHg n = 5, MeHg + Tea n = 5). Different letters indicate significant treatment group differences at $p < 0.05$. 
Figure 3.9  NMDA receptor binding (fmol/mg protein) ± SEM in dams (control $n = 5$, Tea $n = 5$, MeHg $n = 5$, MeHg + Tea $n = 5$).
Figure 3.10  TBARS concentrations ± SEM at PND 14 (control n = 5, Tea n = 5, MeHg n = 6, MeHg + Tea n = 6) and PND 21 (control n = 5, Tea n = 5, MeHg n = 5, MeHg + Tea n = 5) in pups’ A) serum (nmol/ml), B) cerebrum (nmol/mg), and C) cerebellum (nmol/mg). Averaged male and female littermate values are presented. Different letters indicate significant treatment group differences within each PND age at $p < 0.05$. 
3.3.6 Liver cytochrome activation

Although in vitro data suggest that R. tomentosum extract may affect the activity of various cytochrome p450 isoforms (Tam et al., 2009), in this in vivo perinatal exposure scenario there were no treatment effects on BROD, EROD and PROD activities in the liver of PND 14 or 21 pups (data not shown).

3.4 Discussion

The phytochemical composition and in vitro antioxidant properties of Rhododendron tomentosum ssp. subarcticum, a medicinal plant traditionally consumed by Inuit populations, have been previously characterized (Black et al., 2011, submitted). Among the most abundant phytochemicals identified were (+)-catechin, quercetins, and procyanidins, which are all well characterized plant-derived antioxidants able to protect against oxidative stress in vivo (Ghosh et al., 2010, Lee et al., 2003, Zdunic et al., 2009). Knowing that other plant extracts have been shown to mitigate MeHg-induced oxidative stress and neurotoxicity in adult rodents (Lucena et al., 2007; Farina et al., 2005), we chose a rat perinatal exposure model to compare the effects on health and neurodevelopment of MeHg administered alone or in combination with R. tomentosum extract.

Perinatal exposure to MeHg administered alone significantly impaired pup growth and survival, increased lipid peroxidation in serum and NMDA receptor levels in brain, decreased grip strength, caused hyperactivity and loss of central aversion in open field testing, and delayed acoustic startle response time. MeHg had no affect on the timing of
eye and ear opening and on GSH concentration in serum, cerebellum and cerebrum. The
growth, behavioural and oxidative stress findings were consistent with the published
literature on the effects of MeHg (Johansson et al., 2007; Pelletier et al., 2009; Beyrouty and
Chan 2006; Farina et al., 2004). This is the first time perturbed NMDA-Rs have been noted
following developmental MeHg exposure and the mitigation of MeHg-induced NMDA-R
elevation by a plant-based antioxidant.

Exposure to MeHg in adult animals has been correlated to a decrease in NMDA-R
binding, which might be considered an adaptive response to the MeHg-induced elevation of
synaptic glutamate levels (Basu et al., 2007). In our perinatal exposure setting, MeHg
administration was associated with increased NMDA-R binding in PND 21 pups. Data on the
effects of MeHg on NMDA-R levels in the developing brain are scarce, but others have also
reported elevated NMDA-R protein levels following developmental MeHg exposure in mice
(Gao et al., 2008). Elevated NMDA-Rs were also detected in a human neuroblastoma cell
culture experiment following MeHg exposure (Ndountse and Chan, 2008). The
 glutamatergic system plays a crucial role in the developing brain where NMDA-R subunit
composition is temporally and regionally regulated (Farrant et al., 1994; Monyer et al.,
1994). NMDA-R do not reach functional maturity until a few weeks after birth (Haberny et
al., 2002; Sircar, 2003) and are much more sensitive to in vitro MeHg inhibition of substrate
binding than adult brain extracts (Rajanna et al., 1997). For example, the neonatal NMDA-Rs
are primarily composed of NR1 and NR2B subunits, whereas adult NMDA-Rs are composed
on NR1 and NR2A or NR2C subunits. The NR2B subunit does not have the ability for Mg^{2+}
gating, which renders neonatal NMDA-Rs more prone to excitotoxicity (Haberny et al., 2002). Hence, a very different and dynamic cellular context may explain the divergent responses observed in developing and mature brains.

Other than a single instance of decreased lipid peroxidation in PND 21 pups’ cerebrum, exposure to Tea alone had no effect on any of the endpoints measured in the pups. However, MeHg and Tea co-exposure altered several endpoints. Tea supplementation may have altered MeHg pharmacokinetics: THg and MeHg concentrations were generally higher and the relative proportion of MeHg was lower in pup’s tissues of the MeHg + Tea treatment group. Surprisingly, the increased tissue mercury levels in the MeHg + Tea animals did not add to the adverse effects of MeHg on pup development and neurobehaviour, as Tea co-administration did not affect MeHg’s impact on pups growth and mortality, grip strength, ambulatory behaviour in open field testing, and acoustic startle response latency. However, Tea co-exposure abolished MeHg effect on brain NMDA receptor binding and either exacerbated (PND 14 cerebellum) or antagonized (PND 21 serum) MeHg effects on lipid peroxidation. Finally, although exposure to MeHg or Tea administered alone had no effect on the timing of eye opening, co-exposure resulted in precocious eye opening (Fig. 3.2). This observation has also been reported following MeHg co-exposure with selenium and vitamin E (Beyrouty and Chan, 2006) and polychlorinated biphenyl 126 (Vitalone et al., 2008). However, the etiology, significance and consequences of this phenomenon remain obscure.
Most reports describing animal exposure to purified phytochemicals or phytochemical extracts generally attributed beneficial effects at least in part to their antioxidant properties (Ghosh et al., 2010; Khalatbary et al., 2010). Although *R. tomentosum* extract exhibited high *in vitro* antioxidant potency, (Black et al., 2011, *submitted*) our observations in developing rat pups at PND 14 and 21 revealed a more complex situation. In the cerebrum, exposure to Tea administered alone or in combination with MeHg lowered lipid peroxidation below levels observed in the control group at PND 21, while it had no effects at PND 14. Results were more surprising in PND 14 cerebellum, where both Tea and MeHg produced a non-significant increase in lipid peroxidation when administered separately, and a significant 3-fold increase when administered together. Exposure to MeHg significantly increased lipid peroxidation in serum at both PND 14 and 21, and Tea co-exposure abolished MeHg-induced increase in lipid peroxidation only at PND 21. Overall, Tea co-exposure potentiated the effect of MeHg on lipid peroxidation at PND 14 and antagonized it at PND 21. Several possible mechanisms, such as different concentrations of mercury and phytochemicals at PND 14 and PND 21, may explain these observations. Interestingly, the therapeutic dose-range of phytochemicals can be narrow (Ossola et al., 2009) and phytochemical potentiation of MeHg effects on lipid peroxidation has also been reported in adult mouse cerebellum (Martins et al., 2009).

Perinatal exposure to MeHg resulted in hyperactivity and a trend of thigmotaxis loss (preference for the proximity of the surrounding walls) in open field testing. A loss of thigmotaxis may indicate a loss of anxiolytic activity (Treit and Fundytus, 1998). These
observations are commonly reported following rat exposure to NMDA-R antagonists (Koros et al., 2007; Adamec et al., 1999; Plaznik et al., 1994) and interestingly, MeHg exposure also affected NMDA-R binding in vitro. In our study, Tea counteracted MeHg’s effect on NMDA-R binding but not on open field hyperactivity or thigmotaxis, which suggests a more complex relationship between NMDA-R perturbation and spontaneous behaviour.

THg concentrations measured in MeHg + Tea exposed pup tissues were generally higher than those measured in MeHg-treated pups, although this trend was only statistically significance in PND 21 blood. Pup growth curves were very similar in these two treatment groups, ruling out dilution by growth as a potential explanation for differing mercury body burdens. Although not statistically different from the MeHg treatment group, the lower mortality rate observed in the MeHg + Tea treatment group may have resulted in the survival of more heavily contaminated pups, resulting in higher average mercury concentrations.

Alternatively, it is also possible that Tea administration altered MeHg bioavailability. MeHg ingested in food or excreted in bile is efficiently absorbed through intestinal membranes, a process which may be affected by dietary nutrients (Chapman and Chan, 2000). Intestinal bacteria can demethylate MeHg into inorganic mercury which is poorly absorbed, thus breaking the enterohepatic cycling of MeHg and favouring elimination in feces. Higher mercury body burdens observed following co-administration of MeHg and antibiotics clearly supports the physiological relevance of this mechanism (Rowland, 1988). Phytochemicals can have bactericidal or bacteriostatic properties (Cowan, 1999) and may
even modulate intestinal bacterial populations (Lee et al., 2006; Tzounis et al., 2008). It is therefore tempting to speculate that alteration of the gut flora by Tea may have affected demethylation rates, resulting in altered MeHg pharmacokinetics and bioavailability. Further investigations will be needed to appropriately test this hypothesis.

Inorganic mercury can persist longer than MeHg in rat tissue (Farris et al., 1993). All of the mercury present in blood and brain of the MeHg-treated pups at PND 14 was the unmetabolized MeHg, while in the MeHg + Tea treatment group, MeHg represented only 84 ± 9% and 85 ± 8% of the total Hg measured in blood and brain, respectively. However, this trend only reached statistical significance in blood at PND 21. Rates of in vitro demethylation of MeHg in liver slices and cultured astrocytes can be accelerated by the presence of reactive oxygen species (Shapiro and Chan, 2008; Yasutake and Hirayama, 2001). Thus it is possible that the MeHg-induced oxidative stress by Tea observed at PND 14 may have favoured MeHg demethylation. However, it is worth noting that alteration of MeHg bioavailability either by diet or antibiotic administration also affected the ratio of THg to MeHg in rat tissues (Rowland et al., 1984, Rowland, 1988).

3.5 Conclusion

In summary, perinatal exposure to *R. tomentosum* extract administered alone had very little effect on pups health and development, but it was nevertheless able to modulate the developmental toxicity of MeHg, such as the brain NMDA-R and serum lipid peroxidation levels. However, evaluation of co-exposure effects was dependant on the
developmental stage and endpoints assessed, as Tea co-administration had no significant
effects on most MeHg-induced perturbations, counteracted or potentiated MeHg’s effects
on other endpoints and affected new, previously undisturbed parameters. The molecular
mechanisms of MeHg toxicity are still incompletely characterized, and our knowledge of the
possible interactions with nutrients is even more rudimentary. Our results illustrate the
complex nature of contaminant-nutrients interactions and advocate for careful
consideration of potentially beneficial and harmful effects of diet on contaminant toxicity.
With a more thorough knowledge of the molecular mechanisms underlying these
interactions, better human health risk assessment and dietary advices for the mitigation of
MeHg toxicity can be achieved.
CHAPTER FOUR

Molecular outcomes of developmental methylmercury exposure and Northern Labrador Tea (Rhododendron tomentosum ssp. subarcticum) co-exposure in rats.

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5. Provided laboratory resources and expertise for plant extraction. Centre for Advanced Research in Environmental Genomics (CAREG), University of Ottawa.

6. Provided laboratory resources and expertise for protein immunoblot analysis. Centre for Advanced Research in Environmental Genomics (CAREG), University of Ottawa.

7. Contributed to original ideas, supervised Western Blot analysis, and revised the manuscript. Hazard Identification Division, Environmental Health Science and Research Bureau, Health Canada.
4.1 Introduction

DNA microarray technology is a powerful tool for examining the expression of thousands of genes simultaneously and has contributed towards the advancement of multiple areas of research including pharmacology, oncology, and toxicology. The introduction of the use of DNA microarrays within toxicology, “toxicogenomics”, was embraced as a method to reduce animal use and improve data collection (Lovett, 2000). The ability of toxicogenomics to detect patterns of transcriptional alterations in large-scale datasets has allowed the discovery of novel biomarkers of contaminant exposure (Tugwood et al., 2003) and the elucidation of previously unknown cellular mechanisms or adaptive genomic responses of toxicity (Nuwaysir et al., 1999).

The wide range of perturbations reported in the literature suggests that no single molecular mechanism is responsible for the severity of effects observed following developmental MeHg exposure. It appears that astrocytes play a role in two of the best-characterized MeHg induced perturbations within the central nervous system (CNS); glutamate imbalance and oxidative stress. Evidence suggests that MeHg blocks the uptake of glutamate by astrocytes, resulting in an accumulation of excitatory neurotransmitter in the synaptic cleft and excitotoxicity of surrounding neurons (Allen et al., 2002). Excitotoxicity is characterized by \( \text{Ca}^{2+} \) imbalance, nitrotyrosine release, oxidative stress and apoptosis (Michaelis, 1998). The N-methyl-D-aspartate receptor (NMDA-R) appears to play a significant role in MeHg-induced excitotoxicity (Cagiano, et al 1990; Juarez et al., 2005;
Oxidative stress can also be produced independent of excitotoxicity. MeHg may enter the mitochondria membrane and disrupt the electron transport chain releasing reactive oxygen species (ROS) into the cytoplasm (Gasso et al., 2001). The ability of the cell to respond to oxidative stress is also hindered by the MeHg-induced inhibition of cysteine uptake, a crucial amino acid for the production of the endogenous antioxidant, reduced glutathione (GSH) (Allen et al., 2002). Glutathione exists in a reduced form (GSH) and oxidized form (GSSG), and it is the balance of GSH:GSSG that maintains a low oxidative state in healthy tissues (Monks et al., 1999). GSH also binds to MeHg aiding in cellular export and detoxification (Rooney, 2007).

Furthermore, CNS oxidative stress is associated with multiple neurodegenerative diseases, such as Alzheimer’s and Parkinson’s (Dorszewska et al., 2007). The use of microarray technology for the identification of impacted genes and pathways is well suited to the study of MeHg, a toxicant with multiple suggested molecular targets and a diversity of health outcomes.

Previous studies have attempted to elucidate the underlying mechanisms of MeHg toxicity with the use of microarray technology (Ayensu and Tchounwou, 2006), although only a few have focused on developmental neurotoxicity (Padhi et al., 2008; Glover et al., 2009; Shimada et al., 2010). Padhi et al. (2008) found MeHg to disrupt the expression of genes responsible for cellular metabolism, signal transduction, and cell differentiation in PND 14 male rat pups. The other two studies, which utilized mice perinatal exposure experiments, found MeHg to disrupt the expression of genes responsible for transcription
regulation, zinc-binding, and cytoskeleton (Glover et al., 2009), or cardiovascular system, organismal development, and protein synthesis (Shimada et al., 2010). Counter to well-accepted hypotheses of MeHg neurotoxicity, these studies did not detect an enrichment of gene expression related to pathways for antioxidant response or glutamate regulation.

Detecting transcriptional changes within the brain can be especially challenging as gene expression is under tight control and genes of interest often have low RNA yields (Newton et al., 2005). Furthermore, the multiple testing correction methods required to minimize the false detection rate (type I errors) of significantly altered genes becomes progressively more stringent as the size of the dataset increases, which may result in the loss of biologically relevant signals (type II errors). To address these limitations, neurotoxicity studies often use a reduced fold-change cut-off criterion, customized arrays with fewer probes (Newton et al., 2005), or less conservative multiple correction statistical analysis (Shimada et al., 2010). In fact, it has been demonstrated that more reliable and reproducible results are obtained by using a data analysis approach that includes some preliminary data quality control followed by a fold change ranking cut-off criteria compared to multiple correction analysis (Shi et al., 2008).

MeHg co-exposure studies provide further evidence for the role of glutamate excitotoxicity and oxidative stress in MeHg-induced neurotoxicity. Plant derived phytochemicals have repeatedly demonstrated protection against NMDA-R mediated excitotoxicity and oxidative stress in vivo and in vitro (Silva et al., 2008; Campos-Esparza et al., 2009; Chen et al., 2008; Cho and Lee, 2004), and mitigation of MeHg-induced
neurotoxicity (Lucena et al., 2007; Yongjin et al., 2008; Farina et al., 2005). Phytochemicals may exert their protective actions through the alteration of MeHg toxicokinetics (Chapman and Chan, 2000), reduction of cellular oxidative stress, activation of enzymatic antioxidant systems, restoration of the mitochondrial membrane potential, and/or modulation of cell signalling pathways (Campos-Esparza et al., 2009).

The purpose of our study was to elucidate the underlying mechanisms of MeHg-induced developmental neurotoxicity and to evaluate the ability of a dietary antioxidant to interfere with these molecular endpoints. We used Northern Labrador Tea (Rhododendron tomentosum ssp. subarcticum) ethanol extracts (Tea) as our dietary antioxidant since its free radical scavenging properties are well characterized (Black et al., 2011, submitted). Rats were dosed throughout gestation and lactation and the resultant gene transcriptional changes in the cerebellum of PND14 male rats were assessed with DNA microarrays. Rats were dosed with MeHg and/or Tea via diet in contrast to invasive dosing methods such as gavage, which served two purposes; it approximated the most common MeHg exposure scenario in humans, and minimized the impact of handling stress (Branchi et al., 2005) that may alter brain transcriptional profiles. The cerebellum was assessed as it is a known target of MeHg toxicity (Roegge and Schantz, 2006). Ideally several time points and both genders would have been sampled, however we choose to focus on PND 14 males to ensure adequate replicate sampling and for interstudy comparison (Padhi et al., 2008).
4.2 Methodology

4.2.1 Animals and dosing

Please refer to section 3.2.1 and 3.2.2.

4.2.2 RNA extraction

Total RNA was extracted from pup cerebellum using TRIzol reagent (Invitrogen, Burlington, Canada) and purified with RNeasy Mini Kit (Qiagen, Mississauga, Canada) according to manufacturer’s protocols. RNA content was determined with A260/280 optical density ratios, which ranged from 2.0 to 2.1. The RNA integrity was verified by Agilent 2100 Bioanalyzer (Agilent technologies, Mississauga, Canada) and only high quality RNA (28S/18S > 1.8) was used for further analysis. RNA was isolated in batches that contained one sample from each treatment group, and stored at -80°C until use.

4.2.3 Chip hybridization and scanning

Microarray hybridization was performed at the UHN Microarray Centre using the GE-v5_95_Feb07 protocol (University Health Network, Toronto, Canada) in a 2 colour universal RNA rat reference design. Agilent 4 X 44,000 rat oligo arrays (G4131F) were hybridized using the Agilent Low RNA Input Fluorescent Linear Amplification Kit and hybridized using the In Situ Hybridization Kit according to manufacturer’s protocols (Agilent Technologies). Briefly, 500 ng of total RNA from a single rat pup cerebellum or Rat Universal Reference RNA was reverse transcribed and linearly amplified. The amplification product was used in an in vitro transcription reaction to generate cDNA in the presence of cyanine 3-labeled UTP or
cyanine 5-labeled UTP, respectively. Labelled cDNA was fragmented and hybridized to chips incubated at 60°C for 17 hours at 20 rpm, washed and scanned (Agilent G2565BA DNA Scanner). Each microarray slide contained the 4 treatment groups in a staggered position design (5 biological replicates/treatment; 20 samples; 20 arrays on 5 slides). Images were processed and analyzed using Agilent feature extraction software version 9.1.

4.2.4 Protein immunoblot analysis

Protein extracts were prepared from female PND 14 rat pups’ cerebellums (5 biological replicates/treatment; 20 samples). Tissue was homogenized in 10x volume Tris sonification buffer (31 mM Tris-HCl pH 6.8 and 1.5% SDS), sonicated on ice for 30 seconds, and boiled for 10 minutes. Processed samples were then centrifuged at 14,000 G for 10 minutes at 4°C, and the supernatant transferred to a clean tube. Protein concentrations were assayed using the RC DC Protein Assay Kit (Bio-Rad, Mississauga, Canada) according to manufacturer protocol. Samples were diluted to 1.0 mg protein/ml with SDS-PAGE protein loading buffer (31 mM Tris-HCl pH 6.8, 1.5% SDS, 5% glycerol, 2.5% B-mercaptoethanol, 0.1% bromophenol blue). Equivalent amounts of protein (5 µg) were loaded on 12% polyacrylamide gels in such a way that every gel contained each treatment group in a staggered position and a pooled reference sample as a loading control. Proteins were resolved by electrophoresis and transferred to Immobilon-FL PVDF transfer membranes (Millipore, Billerica, USA) according to the manufacturer’s protocols. Membranes were incubated for one hour in blocking solution (TBS + 5% non-fat powdered milk), then with rabbit polyclonal anti-Nurr1 antibody (1 mg/ml) (Santa Cruz Biotechnology, Santa Cruz, USA)
and rabbit polyclonal anti-β-actin antibody (1 mg/ml) (Abcam, Cambridge, USA) for 2 ½ hours at room temperature in a solution of TBST (TBS, 0.1% Tween 20, 1% non-fat powdered milk). Membranes were washed three times for 10 minutes in TBST and then incubated for 55 minutes with goat anti-rabbit IgG Q-dot625 secondary antibody (1 µM) (Invitrogen). Membranes were washed three times for 10 minutes in TBST and visualized by UV illumination on a Gel-Doc XR imaging station using a 580 nm large band pass (120 nm) filter. Intensity values for Nurr1 and β-actin bands were quantified and normalized according to the pooled reference sample using Quantity One 1-D Analysis software (Bio-Rad). Each biological replicate was run in quadruplicate and the relative intensity ratio was averaged.

4.2.5 Data analysis

Microarray median signal intensities were analysed by a block design (Kerr and Churchill, 2001). The background fluorescence was measured using the Agilent negative controls “(-)3xSLv1”. Probes with signal intensities less than the trimmed mean (trim = 5%) plus three trimmed standard deviations of the negative controls were flagged as absent within the background signal. Probes were flagged as present if there was at least one experimental condition that had at least 4 samples (observations) with signal intensities above background. Raw data was normalized using LOWESS. Ratio intensity plots and heat maps for the raw and normalized data were constructed to identify outliers. Three samples from the same microarray were removed from the analysis due to poor data quality and final analysis included 17 biological replicates on 5 4X4 microarray slides. Log2 of the relative
intensities was read into R (R Foundation for Statistical Computing, Vienna, Austria, 2010) and MAANOVA library was used to detect differential gene expression using treatment as the main effect. The test statistic using the James-Stein shrinkage variance estimator was used to test for treatment effects (Cui et al., 2005). The \( p \)-values for all statistical tests were estimated by the permutation method using residual shuffling, followed by adjustment for multiple comparisons using the false-discovery rate (FDR) approach (Benjamini and Hochberg, 1990). The fold change calculations were based on the least-square means. Significant genes were identified as having an adjusted \( p < 0.05 \) for any individual contrast.

Due to the small size of the generated gene list (FDR \( p < 0.05 \); fold change \( \geq 1.3 \); 3 annotated genes), we decided to data mine our results with less stringent cut-off values. Gene lists were created for ontology annotation and hierarchical clustering analysis where significant genes were identified as having an unadjusted \( p < 0.005 \) and fold change \( \geq 1.3 \), which produced 84 annotated signals. Additional gene lists were created for gene ontology (GO) and molecular pathway analysis where significant genes were identified as having a \( p < 0.1 \) and fold change \( \geq 1.3 \), which produced 1255 total signals. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to conduct GO category enrichment analysis and pathway analysis using the Kyoto Encyclopedia of Genes and Genomes (KEGG) (Huang et al., 2009; Dennis et al., 2003). Gene annotations were expanded using NCBI Entrez Gene ID, Unigene, BLAST, and Pubmed. Transcribed sequences and expression sequence tags (ESTs) that could not be identified were eliminated from analysis.
Hierarchical cluster analysis was performed with Pearson correlations on the log$_2$-transformed expression ratios of selected gene lists ($p < 0.005$; fold change $\geq 1.3$).

Nurr1:β-actin protein signal intensity ratios obtained from immunoblotting were assessed with SPSS version 15 software (SPSS Inc., Chicago, USA). Treatment group differences were determined with one-way ANOVAs followed by Tukey post hoc test when appropriate. Differences were considered significant when $p < 0.05$.

4.3 Results

Out of 43,894 probes on the 44k oligo arrays there were 40,028 probe signals with raw intensities flagged as “present”. Among the 40,028 probes, MAANOVA adjusted for multiple comparisons (FDR $p < 0.05$) indentified 11 known genes that were differentially expressed between one or more treatment groups. Of these 11 genes, 3 had a fold change $\geq 1.3$. When we assessed the gene expression dataset using the non-FDR MAANOVA ($p < 0.005$) we identified 84 known genes that were differentially expressed with at least a 1.3 fold change in one or more treatment groups.

4.3.1 Hierarchical cluster analysis

Hierarchical cluster analysis on array samples correctly separated 14 of the 17 arrays into 4 distinct nodes representing the 4 treatment groups based on the differential gene expression pattern compared to the control animals ($p < 0.005$; fold-change $\geq 1.3$; 84 genes) (Fig. 4.1). Of the three arrays that did not cluster correctly into a treatment group node, one control sample (array 1) clustered outside of the 4 nodes, and two samples were
Figure 4.1 Hierarchical cluster analysis of array samples (n = 17) and genes (n = 84) whose expression was altered more than 1.3 fold compared to the control group (p < 0.005). Vertical tree branches; Control (red), MeHg (yellow), Tea (light blue), MeHg + Tea (dark blue). The heat map colour scale represents gene expression compared to control; red (up regulated), black (no change), and green (down-regulated).
incorrectly placed within the control node; MeHg (array 10) and MeHg + Tea (array 5). Array 5 was on the same slide as the 3 arrays that did not meet the quality control inclusion criteria and were excluded from the analysis. Given that the pups were indirectly dosed and an out-bred strain of rats was used, we were satisfied with the clustering.

**4.3.2 Principal component analysis**

Further quality control of expression results was provided by principal component analysis (PCA) of the differentially expressed genes ($p < 0.005$; fold-change $\geq 1.3$; 84 genes) (Fig. 4.2). The 3 exposure groups were separated from the control group with minor overlaps occurring in each group. The MeHg + Tea treatment group separated out in between the individual MeHg and Tea treatment groups. Interestingly, the MeHg treatment group appeared to have the greatest variance, suggesting a high individual-specific genomic response. A total of 59.1% of the observed variance was accounted for by the 3 principal components: the 1$^{\text{st}}$ component accounted for 29.5%; the 2$^{\text{nd}}$ component accounted for 17.8%; the 3$^{\text{rd}}$ component accounted for 11.8% of variance.

**4.3.3 Treatment effects on gene expression**

There were no differentially expressed genes (FDR $p < 0.05$) in common between treatment groups (Fig. 4.3). Using the multiple testing correction, a total of 3 differentially expressed genes were detected with a fold-change equal to or greater than 1.3 compared to control: MeHg group had 2 up-regulated genes ($Ppp1r7$, $Ppp2r5c$), and MeHg + Tea group had 1 up-regulated gene ($Nmral1$) (Table 4.1). $Ppp1r7$ and $Ppp2r5c$ are highly conserved
Figure 4.2 Principal component analysis of the relationship between the gene expression resulting from vehicle control (▲), MeHg (■), Tea (X), MeHg + Tea (●) exposure. Analysis was conducted with differentially expressed genes whose expression was altered at least 1.3 fold compared to the control group ($p < 0.005$) in array samples ($n = 17$). X-axis (1st component) accounted for 29.5% of variance; Y-axis (2nd component) accounted for 17.8% of variance; Z-axis (3rd component) accounted for 11.8% of variance; cumulated axes accounted for 59.1% of variance.
Figure 4.3  Venn diagram depicting number of genes significantly different between treated and control pups using a gene list with multiple testing corrections (FDR $p < 0.05$; fold change $\geq 1.3$).
Table 4.1  Differentially expressed genes in PND 14 male cerebellum following developmental exposure to MeHg, Tea, and MeHg + Tea (FDR $p < 0.05$; Fold change $\geq 1.3$).

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene Name</th>
<th>Gene Symbol</th>
<th>Biological function</th>
<th>MeHg</th>
<th>Tea</th>
<th>MeHg + Tea</th>
</tr>
</thead>
<tbody>
<tr>
<td>NM_001009825</td>
<td>Protein phosphatase 1, regulatory (inhibitor) subunit 7</td>
<td>Ppp1r7</td>
<td>Tumour suppression, protein folding, enzyme regulation</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>NW_047762</td>
<td>Protein phosphatase 2, regulatory subunit B', gamma subunit</td>
<td>Ppp2r5c</td>
<td>Protein binding, negative control in cell growth and division</td>
<td>↑</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>XM_213217</td>
<td>NmrA-like family domain containing 1</td>
<td>Nmral1</td>
<td>Regulation of nitrogen, oxidation reduction</td>
<td>-</td>
<td>-</td>
<td>↑</td>
</tr>
</tbody>
</table>
genes part of the phosphoprotein phosphatase family, which are involved in protein post-translational modifications. Specifically, \textit{Ppp1r7} is a tumour suppressor gene implicated with human carcinogenesis (Harima et al., 2009), and \textit{Ppp2r5c} is a biomarker for progressive B-cell chronic lymphocytic leukemia disease (Falt et al., 2005). Since these genes are commonly down-regulated in cancer patients or cancerous cell lines, it is possible that the observed up-regulation in MeHg treated animals may be indicative of an inhibition of cell cycle progression and cellular proliferation. \textit{Ppp1r7} and \textit{Ppp2r5c} were not significantly affected by MeHg + Tea co-exposure. On the other hand, \textit{Nmral1} was up-regulated exclusively following MeHg + Tea co-exposure. \textit{Nmral1} codes for a protein crucial for early development (Nunez-Corcuera et al., 2008), and is linked to nitrogen regulation and oxidation reduction.

As discussed previously, the false discovery rate multiple testing correction analysis (FDR) was a highly conservative approach to exclude false positive differentially expressed genes from our large dataset. However this analysis may have excluded biologically relevant genes affected by exposure or co-exposure. In order to further explore the dataset we also used the non-FDR MAANOVA ($p < 0.005$) to identify 102 differentially expressed probes with at least a 1.3 fold change in one or more treatment groups. Of these 102 probes, 84 were known genes with annotation (Table 4.2). The co-exposure group of MeHg + Tea had 35 differentially expressed genes (17 up-regulated, 18 down-regulated), followed by the MeHg exposure group with 29 genes (19 up-regulated, 10 down-regulated), and Tea exposure group with 22 genes (10 up-regulated, 12 down-regulated).
Table 4.2  Differentially expressed genes in PND 14 male cerebellum following developmental exposure to MeHg, Tea, and MeHg + Tea ($p < 0.005$; fold change $\geq 1.3$).

<table>
<thead>
<tr>
<th>Accession</th>
<th>Gene</th>
<th>Gene Symbol</th>
<th>Biological function</th>
<th>MeHg</th>
<th>Tea</th>
<th>MeHg + Tea</th>
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<td>TC557877</td>
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<td>TC566391</td>
<td>Prostaglandin E receptor 4, subtype EP4</td>
<td>Ptger4</td>
<td>Binds prostaglandin estradiol (PGE(2) and induces cAMP-dependent bone reabsorption</td>
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<tr>
<td>XM_001067682</td>
<td>Ligase IV, DNA, ATP-dependent</td>
<td>Lig4</td>
<td>Cellular metabolism</td>
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<td>TC529062</td>
<td>Zinc finger protein 74</td>
<td>Zfp74</td>
<td>DNA-dependent regulation of transcription</td>
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<td>NM_031745</td>
<td>CAP-GLY domain containing linker protein 1</td>
<td>Clip1</td>
<td>Linker protein that associates with growing ends of microtubules</td>
<td>↓</td>
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<td>AW917588</td>
<td>Splicing factor, arginine/serine-rich 5</td>
<td>Sfrs5</td>
<td>Regulator of alternative pre-mRNA splicing; may play a role in cell cycle regulation</td>
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<td>DQ268830</td>
<td>Neuron-derived orphan receptor</td>
<td>Nr4a3</td>
<td>Transcription regulation, neuronal &amp; auditory development</td>
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<td>AA900367</td>
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<td>NM_017306</td>
<td>Dodecenoyl-Coenzyme A delta isomerase</td>
<td>Dci</td>
<td>Lipid metabolism</td>
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<td>Plscr4</td>
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<td>A_44_P250371</td>
<td>Sorbitol dehydrogenase</td>
<td>Sord</td>
<td>Catalyzes the metabolism of L-sorbose in the polyol pathway</td>
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<td>XM_342494</td>
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<td>Bub1b</td>
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<td>Cdk4</td>
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<td>Notch1</td>
<td>Cell morphogenesis, embryonic development, neuron development</td>
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<td>LIAMER</td>
<td>Activates estrogen receptor</td>
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<td>Reg3a</td>
<td>Acute inflammatory response, defence response, response to wounding</td>
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<td>TIMP metallopeptidase inhibitor 3</td>
<td>Timp3</td>
<td>Induction of apoptosis, response to nutrient levels, response to wounding</td>
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<td>Pold1</td>
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<td>Rnpc1</td>
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<td>Lrp4</td>
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<td>Oat</td>
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<td>Ppia</td>
<td>Cellular metabolism</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>BQ211716</td>
<td>Nucleoside diphosphate type motif 3</td>
<td>Nudt3</td>
<td>Diphosphoinositol-polyphosphate diphosphatase activity</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>AI176590</td>
<td>Ring finger protein 19A</td>
<td>Rnf19a</td>
<td>Localized in Lewy bodies, a characteristic neuronal inclusion in Parkinson's disease.</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>AI058940</td>
<td>Microtubule-associated protein RP/EB family member 2</td>
<td>LOC</td>
<td>Microtubule binding</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>NM_022958</td>
<td>Phosphoinositide-3-kinase, class 3</td>
<td>Pik3c3</td>
<td>Proteolysis, response to nutrient levels</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>NM_019163</td>
<td>Presenilin 1</td>
<td>Psen1</td>
<td>Signal transduction, Alzheimer's disease</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>NM_013221</td>
<td>HMG-box transcription factor 1</td>
<td>Hbp1</td>
<td>Transcription regulation</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>AW140602</td>
<td>Cytochrome c oxidase, subunit VIIc</td>
<td>Cox7c</td>
<td>Transmembrane transfer of solutes</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
<tr>
<td>XM_001065112</td>
<td>Proteasome macropain assembly chaperone 3-like</td>
<td>LOC</td>
<td>Unknown</td>
<td></td>
<td>↑</td>
<td></td>
</tr>
</tbody>
</table>
**Figure 4.4** Venn diagram depicting number of genes significantly different between treated and control pups using a gene list without multiple testing corrections ($p < 0.005$; fold change $\geq 1.3$).
Of these 84 differentially expressed genes, only 2 genes were significantly affected in more than one treatment group (Fig. 4.4). One gene (Dci) was in common between the MeHg and MeHg + Tea treatment groups and the other gene (Map3k8) was in common between the Tea and MeHg + Tea treatment groups. Dci codes for dodecenoyl-coenzyme A delta isomerise and is responsible for lipid metabolism (Huang et al., 2009). Map3k8 codes for mitogen-activated protein kinase 8, which is responsible for cell differentiation signalling pathways and is associated with a cellular stress response (Huang et al., 2009). It is interesting to note the small number of genes in common between the exposure and co-exposure treatment groups, a phenomenon observed regardless of the stringency of the statistical analysis.

4.3.4 Gene Ontology category analysis

To assess the functional groups of the differentially expressed genes (p < 0.1; fold change ≥ 1.3) following treatment exposure we used gene ontology (GO) category analysis. The gene expression profile from MeHg exposure was associated with 8 GO categories including those related to translation (GO:6414, 6412), cell cycle (GO:7049, 51301, 0279, 0087), and DNA metabolism (GO:6259, 6260). The expression profile from Tea exposure was also associated with 2 GO categories, both related to the translation categories (GO:6414, 6412). The expression profile from MeHg + Tea exposure was not associated with any GO categories at the FDR < 0.05 cut off (Table 4.3).
<table>
<thead>
<tr>
<th>Gene List</th>
<th>GO Category</th>
<th>% genes in category</th>
<th>Fold Enrichment</th>
<th>p-value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeHg</td>
<td>GO:0006414~translational elongation</td>
<td>4.1</td>
<td>7.8</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>GO:0007049~cell cycle</td>
<td>7.8</td>
<td>3.1</td>
<td>0.000</td>
<td>0.001</td>
</tr>
<tr>
<td></td>
<td>(609 genes, 229 annotated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GO:0006412~translation</td>
<td>7.2</td>
<td>3.1</td>
<td>0.000</td>
<td>0.003</td>
</tr>
<tr>
<td></td>
<td>GO:0006259~DNA metabolic process</td>
<td>6.4</td>
<td>3.3</td>
<td>0.000</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>GO:0051301~cell division</td>
<td>4.1</td>
<td>5.0</td>
<td>0.000</td>
<td>0.007</td>
</tr>
<tr>
<td></td>
<td>GO:0000279~M phase</td>
<td>4.3</td>
<td>4.6</td>
<td>0.000</td>
<td>0.009</td>
</tr>
<tr>
<td></td>
<td>GO:0006260~DNA replication</td>
<td>3.5</td>
<td>5.1</td>
<td>0.000</td>
<td>0.041</td>
</tr>
<tr>
<td></td>
<td>GO:0000087~M phase of mitotic cell cycle</td>
<td>3.2</td>
<td>5.5</td>
<td>0.000</td>
<td>0.048</td>
</tr>
<tr>
<td>Tea</td>
<td>GO:0006414~translational elongation</td>
<td>7.2</td>
<td>15.6</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>GO:0006412~translation</td>
<td>10.3</td>
<td>5.0</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>(452 genes, 114 annotated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GO:0006397~mRNA processing</td>
<td>4.1</td>
<td>4.0</td>
<td>0.004</td>
<td>5.553</td>
</tr>
<tr>
<td></td>
<td>GO:0006396~RNA processing</td>
<td>5.1</td>
<td>2.9</td>
<td>0.007</td>
<td>10.092</td>
</tr>
<tr>
<td></td>
<td>GO:0016071~mRNA metabolic process</td>
<td>4.1</td>
<td>3.5</td>
<td>0.008</td>
<td>11.800</td>
</tr>
<tr>
<td></td>
<td>GO:0022613~ribonucleoprotein complex biogenesis</td>
<td>3.1</td>
<td>4.8</td>
<td>0.008</td>
<td>12.024</td>
</tr>
<tr>
<td></td>
<td>GO:0022618~ribonucleoprotein complex assembly</td>
<td>2.1</td>
<td>8.2</td>
<td>0.013</td>
<td>18.554</td>
</tr>
<tr>
<td>MeHg + Tea</td>
<td>GO:0008015~blood circulation</td>
<td>4.6</td>
<td>5.2</td>
<td>0.002</td>
<td>3.737</td>
</tr>
<tr>
<td></td>
<td>GO:0000902~cell morphogenesis</td>
<td>6.5</td>
<td>3.4</td>
<td>0.003</td>
<td>4.527</td>
</tr>
<tr>
<td></td>
<td>(328 genes, 106 annotated)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>GO:0031667~response to nutrient levels</td>
<td>5.9</td>
<td>3.6</td>
<td>0.003</td>
<td>5.093</td>
</tr>
<tr>
<td></td>
<td>GO:0009611~response to wounding</td>
<td>7.2</td>
<td>3.0</td>
<td>0.004</td>
<td>6.054</td>
</tr>
<tr>
<td></td>
<td>GO:0009991~response to extracellular stimulus</td>
<td>5.9</td>
<td>3.4</td>
<td>0.005</td>
<td>7.603</td>
</tr>
<tr>
<td></td>
<td>GO:0032989~cellular component morphogenesis</td>
<td>6.5</td>
<td>3.0</td>
<td>0.005</td>
<td>8.579</td>
</tr>
<tr>
<td></td>
<td>GO:0003073~regulation of arterial blood pressure</td>
<td>2.6</td>
<td>10.6</td>
<td>0.006</td>
<td>9.693</td>
</tr>
</tbody>
</table>
Table 4.4  KEGG pathways significantly associated with genes with a differential expression of at least 1.3-fold change in each treatment group ($p < 0.1$).

<table>
<thead>
<tr>
<th>Gene List</th>
<th>Term</th>
<th>Count</th>
<th>% genes in pathway</th>
<th>Fold Enrichment</th>
<th>$p$-value</th>
<th>FDR</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeHg (609 genes, 229 annotated)</td>
<td>Ribosome</td>
<td>14</td>
<td>4.1</td>
<td>7.4</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td></td>
<td>Cell cycle</td>
<td>14</td>
<td>4.1</td>
<td>4.9</td>
<td>0.000</td>
<td>0.005</td>
</tr>
<tr>
<td></td>
<td>DNA replication</td>
<td>7</td>
<td>2.0</td>
<td>8.7</td>
<td>0.000</td>
<td>0.133</td>
</tr>
<tr>
<td></td>
<td>p53 signalling pathway</td>
<td>8</td>
<td>2.3</td>
<td>5.3</td>
<td>0.001</td>
<td>0.779</td>
</tr>
<tr>
<td></td>
<td>Systemic lupus erythematosus</td>
<td>9</td>
<td>2.6</td>
<td>4.4</td>
<td>0.001</td>
<td>1.064</td>
</tr>
<tr>
<td></td>
<td>Mismatch repair</td>
<td>4</td>
<td>1.2</td>
<td>7.9</td>
<td>0.013</td>
<td>13.887</td>
</tr>
<tr>
<td></td>
<td>Focal adhesion</td>
<td>10</td>
<td>2.9</td>
<td>2.2</td>
<td>0.033</td>
<td>31.843</td>
</tr>
<tr>
<td></td>
<td>Progesterone-mediated oocyte maturation</td>
<td>6</td>
<td>1.7</td>
<td>3.0</td>
<td>0.047</td>
<td>42.740</td>
</tr>
<tr>
<td>Tea (452 genes, 114 annotated)</td>
<td>Ribosome</td>
<td>13</td>
<td>6.7</td>
<td>14.1</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>MeHg + Tea (328 genes, 106 annotated)</td>
<td>Mismatch repair</td>
<td>3</td>
<td>2.0</td>
<td>13.4</td>
<td>0.020</td>
<td>20.245</td>
</tr>
<tr>
<td></td>
<td>DNA replication</td>
<td>3</td>
<td>2.0</td>
<td>8.4</td>
<td>0.048</td>
<td>41.968</td>
</tr>
</tbody>
</table>
4.3.5 Pathway analysis

We used the same differentially expressed gene lists ($p < 0.1$; fold change $\geq 1.3$) within the Kyoto Encyclopedia of Genes and Genomes (KEGG) to detect functional pathways significantly associated with the gene profiles resulting from our exposure scenarios; MeHg (8 networks), Tea (1 network), and MeHg + Tea (2 networks) (Table 4.4). Both the MeHg and Tea treatments were associated with the ribosomal pathway, which was not associated with the co-exposure treatment. The 2 networks found with MeHg + Tea co-exposure, mismatch repair and DNA replication, were also found with MeHg exposure, which suggests that the perturbation of these pathways was due to MeHg exposure, and the other 6 pathways affected following MeHg exposure were mitigated with Tea co-exposure.

4.3.6 Nurr1 protein quantification

The *Nr4a2* gene, which codes for Nurr1, a nuclear receptor particularly crucial for neuronal development and function, was down-regulated following MeHg exposure ($p < 0.005$; fold change $\geq 1.3$). Nurr1 was selected for the validation of the microarray expression data with protein immunoblot analysis because of its biological function and hypothesized role in the molecular etiology of MeHg-induced neurotoxicity. We detected a 2.8-fold decrease in Nurr1 protein concentration in the PND 14 cerebells of MeHg treated female pups (Fig. 4.5). In addition, the concentration of Nurr1 matched those of control levels following MeHg + Tea co-exposure. Tea exposure had no effect on Nurr1 concentration. These Nurr1 protein level results support the alteration of *Nr4a* transcription levels, which
Figure 4.5  Treatment effects of vehicle control, MeHg, Tea, and MeHg + Tea, on NURR1 protein concentrations in PND14 female rat pup cerebellum; A) example protein immunoblot of NURR1 and β-actin protein signals, and B) average relative expression intensity of NURR1: β-actin protein following exposure to vehicle control, MeHg, Tea, and MeHg + Tea (n = 16). Different letters indicate significant treatment group differences at p < 0.05.
were down-regulated by 1.6 fold following MeHg exposure, yet not significantly affected by MeHg + Tea co-exposure or Tea exposure.

4.4 Discussion

In this study we used a microarray approach to capture a global snapshot of the cellular response following developmental exposure and co-exposures to MeHg and/or Tea, in order to elucidate the mechanistic pathways of MeHg toxicity and the influence of dietary antioxidants on the transcriptional response to MeHg.

The brain is an organ buffered from drastic genomic changes (Newton et al., 2005), therefore we choose to examine genes that had at least a 1.3-fold change in expression over control animals. FDR multiple testing correction analysis identified 3 genes to be differentially expressed in at least one treatment group compared to control animals; 

*Ppp1r7* and *Ppp2r5c* were up-regulated in MeHg exposed pups, and *Nmral1* was up-regulated with MeHg + Tea exposed pups. Tea did not significantly alter gene expression, and Tea co-exposure was able to return the expression of *Ppp1r7* and *Ppp2r5c* to control levels. *Ppp1r7, Ppp2r5c,* and *Nmral1* are conserved in both humans and rodents. The up-regulation of *Ppp1r7* and *Ppp2r5c,* which are both associated with cancerogenesis, may suggest the MeHg-induced inhibition of the cell cycle in the developing cerebellum.

Although only speculative at this time, an inhibition of cell cycle and division during the development of brain cytoarchitecture could explain previously observed pathophysiological perturbations following developmental MeHg exposure (Roda et al.,
2008). Ppp1r7 and Ppp2r5c were not significantly affected in the MeHg + Tea treatment group, suggesting this perturbation was rescued with co-exposure. On the other hand, Nmral1 was up-regulated exclusively following MeHg + Tea co-exposure, which codes for a protein responsible for nitrogen regulation and oxidation reduction. It is interesting to note that although oxidative stress is a hypothesized mechanism of MeHg toxicity, there were no oxidative stress related genes found in the MeHg exposure group, and only a single gene in the MeHg + Tea exposure group. Although this gene list was extremely small, our results suggest the ability of the Tea to mitigate selected genomic responses to MeHg exposure, whereas its co-exposure with MeHg alters other genes previously unaffected by MeHg. Confirmation of this transcriptional response should be explored within a larger dataset.

Since we detected very few differentially expressed genes with the FDR multiple testing correction analysis, we further explored the dataset with less stringent cut-off criteria ($p < 0.005$; fold change $\geq 1.3$) and detected 84 differentially expressed genes. Interestingly, the MeHg + Tea treatment resulted in more differentially expressed genes than either exposure alone, yet there were only 2 genes in common between any of the treatment groups. Together this suggests the transcriptional response signature following a co-exposure cannot be predicted based on individual exposure scenarios. Such a phenomenon has also been reported in similar MeHg developmental co-exposure studies with other contaminants (Padhi et al., 2008; Pelletier et al., 2009), and other experimental models with contaminant co-exposure scenarios (Bae et al., 2001; Finne et al., 2007; Krasnov et al., 2007; Hendriksen et al., 2007). To our knowledge, this is the first time such a
loss of transcriptional signature has been reported following nutrient-contaminant co-exposure. The potential ability of nutritional status to mask the transcriptional signature of toxicants raises important considerations for risk assessment.

MeHg exposure resulted in the down-regulation of 2 isoforms of nuclear receptors, *Nr4a2* and *Nr4a3*, which were unaffected following MeHg + Tea co-exposure or Tea exposure. These genes are part of the NR4A subfamily of nuclear receptors comprised of three members: *Nr4a1*, *Nr4a2* and *Nr4a3* that code for the protein products Nur77, Nurr1, and Nor-1 respectively (Paulsen et al., 1995). All NR4A members are transcription factors highly conserved in humans and rodent species, which bind to cis-acting DNA elements containing the sequence motif AAAGGTCA (Maheux et al., 2005). Each NR4A gene has a different expression profile and target different down-stream genes following external and internal stimuli (Zetterstrom et al., 1996). Due to the down-regulation of 2 of the 3 genes in a single nuclear receptor subfamily we decided to investigate them further.

Nor-1 was first characterized by its role in neuronal apoptosis (Ohkura et al., 1994), and later for its role in inner ear development (Ponnio et al., 2002), and axonal growth and hippocampus-cell survival (Ponnio and Conneely, 2004). Nor-1 is the earliest detected gene to respond following neuronal stimulation and is thought to be an inducer of neuronal plasticity pathways crucial for the process of learning and memory (Sun et al., 2007). In normal conditions Nor-1 is rapidly up-regulated following neuronal stimulation (Sun et al., 2007), whereas in our study it was down-regulated with developmental MeHg exposure treatment. The expression of the NR4A genes has been linked to the stimulation of NMDA-R
(Dragunow et al., 1996), suggesting that developmental MeHg exposure resulted in the
down-regulation or inhibition of this neuronal pathway.

Nurr1 is highly expressed in ventral dopamergic mid-brain neurons, and is crucial for
neuronal development and maintenance (Kadkhodaei et al., 2009). It is associated with the
KEGG molecular pathway of the progression of Parkinson’s disease (PD) (Fig. 4.6). PD is a
neurodegenerative disease that targets the nigrostriateal dopaminergic neurons,
characterized by inflammation, oxidative stress, and cytokine-dependent neurotoxicity (Lee
et al., 2009), which are also outcomes also associated with MeHg toxicity. Furthermore,
Nurr1 protein levels are reduced by 50% in PD patients (Le et al., 2008), which was similar to
the reduction we detected in our study following developmental MeHg exposure.

Further assessment of molecular function revealed that Nurr1 was involved in the
transcriptional control of vasoactive intestinal peptide (VIP), a protein first identified in
bovine intestinal system, now also known as a potent neuroprotectant involved with
various neuronal damage insults including oxidative stress (Luo et al., 2007). Nurr1
expression is associated with oxidative stress resistance, cell survival, neuronal
differentiation, and brain region-specific gene expression profiles (Sousa et al., 2007).
Furthermore, Nurr1 concentrations increased with exposure to antioxidant vitamins C and
E, which are suggested as a supplement for the treatment of early Parkinson’s disease (Lee
et al., 2008). In our study, Nr4a2 transcriptional levels and Nurr1 protein levels were
significantly depressed following MeHg exposure, and the supplementation of Tea with
MeHg exposure returned them to control levels.
Figure 4.6 KEGG neurodegenerative disorders pathway; the gene NR4A2 (as indicated with a green rectangle) is involved with Parkinson’s Disease (http://www.genome.jp/kegg/)
Although genomic changes are interesting for early detection of phenotypic changes and elucidation of novel biomarkers or molecular mechanisms, these genomic changes do not necessarily imply a phenotypic change. There are many post-transcriptional regulatory mechanisms to modulate mRNA translation and degradation (Karijolich et al., 2010). Thus it is of interest to determine if the transcriptional changes translate into actual protein concentration differences. Here we confirmed a 65% reduction of Nurr1 protein levels in PND 14 pups exposed to MeHg, and the return to control levels with MeHg + Tea co-exposure. Unfortunately microarray analysis was conducted on male brain samples whereas immunoblot analysis was conducted on female brain samples due to a limited amount of material for the large number of tissue analyses conducted (microarray, oxidative stress, brain receptor, THg and MeHg concentrations). Although this imposes a limitation on the interpretation of the results drawn from this study it also suggested the robustness of Nr4a2 down-regulation, which may result in a reduction of Nurr1 protein product in both genders following MeHg exposure. Previous studies conducted at the Hazard Identification Division of Health Canada have demonstrated the gender-specific nature of transcriptional response to contaminant exposure, with few genes being similarly affected in both males and females (Padhi et al., 2008). This finding argues for further investigation on the role of Nurr1 in MeHg-induced developmental neurotoxicity.

In order to get a more comprehensive view of the pathways targeted by MeHg and MeHg + Tea exposure, GO functional category analysis and KEGG pathway analysis were conducted using all the genes with at least a 1.3 fold change in expression (p < 0.1). The
Genomic profile from MeHg exposure was associated with several GO categories; translation, cell cycle, cellular metabolism, and cell division. Similarly, the disruption of cellular metabolism was detected in three previously conducted microarray experiments on developmental MeHg exposure in rodents (Padhi et al., 2008; Glover et al., 2009; Shimada et al., 2010), whereas protein synthesis was only detected in the current study (Table 4.5). Cellular differentiation was detected in all but one study (Shimada et al., 2010). Other functional groups that were highlighted in previous studies but not in the present exposure scenario results were; signal transduction, cytoskeleton, transcription, metal binding and immunoglobulin. These differences may be due to different animal model subjects (rat or mouse), exposure designs (gavage, drinking water, or dietary exposure), duration of exposures (7 weeks, 8 weeks, or 11 weeks), and doses of MeHg (2 mg/KgBW/d, 4.5 mg/KgBW/d, or unknown), which demonstrates the difficulty in translating toxicogenomic results for general risk assessment and the complicated molecular etiology of developmental MeHg neurotoxicity. On the other hand, all three studies including the present, did not detect a perturbation of functional groups related to antioxidant production or glutamate regulation despite the well accepted hypothesis of oxidative stress driven MeHg neurotoxicity (Table 4.5).

Both the GO and KEGG analyses highlighted the MeHg-induced perturbation of translation and the ribosomal pathway. Previous studies have also reported the MeHg-induced disruption of the protein synthesis pathway (Shimada et al., 2010), decreased protein production, reduced ribosomal presence on the rough endoplasmic reticulum.
Table 4.5  Comparisons of detected GO functional categories between three microarray experiments including developmental MeHg exposure in a rodent model (Y = detected, N = not detected).

<table>
<thead>
<tr>
<th>GO functional category detected</th>
<th>Current – (Black et al., 2011) &lt;sup&gt;a&lt;/sup&gt;</th>
<th>(Padhi et al., 2008) &lt;sup&gt;a&lt;/sup&gt;</th>
<th>(Glover et al., 2009) &lt;sup&gt;b&lt;/sup&gt;</th>
<th>(Shimada et al., 2010) &lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cellular metabolism</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
</tr>
<tr>
<td>Cell differentiation</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Signal transduction</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Stimuli response</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>Y</td>
</tr>
<tr>
<td>Cytoskeleton</td>
<td>N</td>
<td>Y</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Protein synthesis</td>
<td>Y</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Transcription</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Metal binding</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Immunoglobulin</td>
<td>N</td>
<td>N</td>
<td>Y</td>
<td>N</td>
</tr>
<tr>
<td>Oxidative stress response</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
<tr>
<td>Glutamate regulation</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
</tr>
</tbody>
</table>

<sup>a</sup> Used a rat developmental exposure model  
<sup>b</sup> Used a mouse developmental exposure model
(Lachapelle et al., 1993), and translational inhibition due to perturbations of amino acylation of tRNA (Cheung and Verity, 1985). However, Tea and MeHg + Tea treatments were also associated with translation GO categories and the ribosomal KEGG pathway, suggesting that this perturbation is not particular to MeHg exposure. Indeed, the global modification of proteins is often reported following exposure to contaminants (Merrick and Bruno, 2004; Liebler, 2002; Moreira et al., 2010). This concept of a general response is supported by our results where although the MeHg and Tea exposure groups both had translation as their primary GO category, they had no differentially expressed genes in common. It is possible that the differentially expressed genes that were detected in our study do not reflect the adaptive molecular response of the animals, but rather a distant downstream consequence of the global system perturbation after 6 weeks of chronic MeHg exposure. It would be interesting for future studies to focus on the genomic response in the days following developmental MeHg exposure.

There were no significantly affected GO functional categories following the MeHg + Tea co-exposure in spite of our results where this treatment resulted in the most differentially expressed genes. However, for data mining purposes, it is interesting to note that several functional groups related to the circulatory system were significantly associated with MeHg + Tea exposure at the uncorrected $p < 0.05$ level (Table 4.3). Similar GO category functional groups related to the circulatory system were also associated with MeHg exposure alone, which suggests that this response is due to MeHg exposure and not specific to co-exposure. For example the MeHg treatment had the following categories:
GO:0008015~blood circulation, count = 8, p = 0.027; and GO:0003073~regulation of systemic arterial blood pressure, count = 4, p = 0.047) (data not shown). A similar study also found cardiovascular system genes and pathways to be associated with MeHg exposure (Shimada et al., 2010). Furthermore, recent preliminary data from research conducted at the Hazard Identification Division of Health Canada suggested the functional alteration of the brain circulatory system in rat pups developmentally exposed to MeHg (G. Pelletier, unpublished data). Other functional groups that met these criteria (p < 0.05) following MeHg exposure included response pathways to nutrients, wounding, and extracellular stimulus, some of which have been previously identified (Shimada et al., 2010). The mismatch repair and DNA replication KEGG pathways were significantly associated with MeHg + Tea co-exposure, which were also present with MeHg exposure. This suggested that these 2 networks were not mitigated with Tea co-exposure, whereas and the other 6 pathways associated with MeHg exposure were ameliorated.

4.5 Conclusions

Tea co-exposure demonstrated the ability to mitigate specific transcriptional perturbations following MeHg exposure, whereas Tea exposure alone affected the expression of other genes that were not affected by exposure to MeHg alone. The loss of the MeHg transcriptional response signature under a nutrient co-exposure scenario presents implications for the prediction of toxicity and the identification of exposure biomarkers. Although the genomic profiles from our microarray experiment in general correctly clustered treatment groups with both hierarchical analysis and PCA, we detected
only 3 differentially expressed genes following exposure to MeHg, Tea or MeHg + Tea (FDR $p < 0.05$; fold change $\geq 1.3$). *Ppp1r7* and *Ppp2r5c*, related genes both involved with cancer progression and tumour suppression, were up-regulated with MeHg exposure. *Nmral1*, a gene involved with oxidation reduction, was up-regulated with MeHg + Tea exposure.

When the results were explored without the false discovery rate multiple testing correction we found 2 related genes *Nr4a2* and *Nr4a3*, coding for nuclear receptors down-regulated with MeHg exposure. These genes are crucial for early neural development and learning. Protein levels of Nurr1, the protein product of *Nr4a2* were confirmed with Western blot analysis to corroborate with the lowered mRNA levels. The GO functional categories associated with MeHg exposure are primarily related to translation or cell cycle. Translation was also associated with tea exposure suggesting this may be a general exposure response. KEGG pathway analysis also confirmed these MeHg-targets. There were no GO functional categories significantly associated with MeHg + Tea exposure, however KEGG analysis revealed mismatch repair and DNA replication to be significantly altered. Clearly the result of MeHg + Tea co-exposure is not the sum of their individual exposures, and although Tea was able to mitigate some MeHg-affected pathways, their co-exposure altered previously unaffected pathways. Our results demonstrate the ability of a plant-based antioxidant to mask the transcriptional signature of MeHg, which raises important considerations for risk assessment and the molecular characterization of this potent neurotoxicant.
CHAPTER FIVE

Mercury exposure levels in Costa Rican populations from fish consumption and the reduction of mercury body burden with an antioxidant-rich diet.

Adapted from: Paleah Black¹, Aylin Castillo², Maria Gutiérrez³, David Lean⁴, Juan Valdés⁵, Donna Mergler⁶, Emmanuel Yumvihoze⁷, Berna van Wendel de Joode⁸. Environmental Research. 2011 (In preparation).

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2. Assisted in obtaining dietary surveys and hair samples, helped develop the pilot project. Atmospheric Chemistry Laboratory (LAQAT), Universidad Nacional, Costa Rica.


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7. Hg analysis in biological samples, Hg testing quality control. Centre for Advanced Research in Environmental Genomics (CAREG), University of Ottawa.

8. Contributed to original ideas, supervised epidemiology study and statistical analysis, and revised manuscript. Central America Institute for Studies on Toxic Substances (IRET), Universidad Nacional, Costa Rica.
5.1 Introduction

Methylmercury (MeHg) is a heavy metal capable of causing systemic toxicity in humans and animals. MeHg inflicts damage to the immune system (Hultman and Hansson-Georgiadis, 1999; Shenker et al., 2002), the cardiovascular system (Virtanen et al., 2007; Fillion et al., 2006; Stern, 2005; Chan and Egeland, 2004), and, most notably, the central nervous system (CNS) (Yokoo et al., 2003; Bellum et al., 2007; Mergler et al., 2007). The CNS is particularly vulnerable to MeHg-induced neurotoxicity if exposure occurs during fetal development (Counter and Buchanan, 2004; Bose-O'Reilly et al., 2010). For example, there are cases of severe fetal poisoning even in the absence of any detectable adverse effects in the mother (Igata, 1993).

Developmental MeHg exposure is primarily associated with poor language and neurocognitive performance, impaired attention, memory and motor functions, and delayed brainstem auditory evoked potentials, which have been identified in a wide variety of populations in Northern Quebec (Weihe et al., 2002), New Zealand (Crump et al., 1998), Poland (Jedrychowski et al., 2006), Faroe Islands (Steuerwald et al., 2000), United States of America (Oken et al., 2005), French Guiana (Cordier et al., 2002), and the Brazilian Amazon (Grandjean et al., 1999). Conversely, early reports from the Seychelles Island study did not detect negative outcomes associated with developmental MeHg exposure and actually found improved neurodevelopment scores associated with the increased maternal Hg-hair concentrations (Myers et al., 1997). In this case, maternal Hg-hair was an indication of fish consumption, which is known to also contain important nutrients required during
development for optimal CNS health. Amongst the multitude of potential confounders that may explain these discrepancies, increasing attention is being focused on the confounding nature of dietary nutrients (Choi et al., 2008). Populations in Northern Canada and the Faroe Islands for example, receive much of their MeHg exposure via the consumption of sea mammals, whereas it is via fish consumption in the Seychelles Islands. Without the adequate corrections for nutrient consumption, a known confounder in epidemiological studies (Choi et al., 2008), the harmful effects of low-dose MeHg exposure may not be detected (Budtz-Jorgensen et al., 2007). The presence of MeHg contamination in fish and seafood presents a difficult public health issue, particularly within the various populations worldwide who rely on it as a primary source of dietary protein.

Costa Rica is a small Central American country with high per capita fish consumption, including imported and local tuna, shark, dolphinfish and other carnivorous fish types (FAO, 2006). As with many developing countries, there are no publically issued fish consumption advisories or food guidelines, nor is there a regulatory food inspection agency. Costa Rica hosts many natural and anthropogenic features linked to environmental Hg release or MeHg production, such as active volcanoes, hydroelectric dams, deforestation, wetlands, and operational open-pit mines (MINAE, 2010). High Hg levels have been detected in samples of Costa Rican coastal sediments and corals, which have been attributed to such activities, as well as agrochemical runoff, industrial waste, and coastal refineries (Guzman and Garcia, 2002). It remains unknown if there is a health risk from human MeHg exposure.
Due to bioaccumulation, carnivorous fish generally have higher MeHg tissue levels than non-carnivorous fish, thus it is possible to maximize nutrient intake and minimize MeHg exposure through healthy fish choices (Burger et al., 2005; Mahaffey et al., 2007). In Canada for example, there are publically issued recommended limits for MeHg intake, local fish consumption advisories, and national food guides that include healthy fish choices (Health Canada, 2007b). Multiple countries have issued provisional tolerable daily intake (pTDI) recommendations for women of reproductive age, such as the MeHg pTDI issued in Canada (0.2 µg/KgBW/d), Japan (0.3 µg/KgBW/d), Netherlands (0.1 µg/KgBW/d), and the USA (0.1 µg/KgBW/d) (WHO, 2008). Not all countries have the resources for such a public health approach. To address MeHg exposure issues occurring in multiple populations throughout the world, the World Health Organization (WHO) has set a recommended limit for hair-Hg concentrations of 2 ppm (2 µg/g) for women of reproductive age and children (WHO, 2008). In accordance with the pTDI guideline, a woman may safely consume one and a half fish meals a week (assuming the average fish MeHg concentration is 0.5 µg/g, a fish meal is 150 g, and a 55 kg woman). Clearly some populations do exceed this limit, particularly those who consume more fish, have a preference for carnivorous fish types, or have a lighter body mass such as children. Over the past decade the recommended limit for MeHg consumption has consistently been lowered as new data surfaced, and recently it has been suggested that there may not be a threshold level for adverse neurotoxicity from MeHg exposure (Rice, 2004).
With fish being an excellent source of nutrition and an integral aspect of many cultures, some research has explored the possibility of reducing the health risks of MeHg exposure from fish consumption rather than reducing fish consumption itself. Dietary nutrients may interact with MeHg toxicity by altering absorption, metabolism, compartmentalization, or excretion (Chapman and Chan, 2000). Selenium has gained particular interest as it is naturally present in fish, has a high affinity for MeHg to form less toxic MeHg-Se compounds, and has potent antioxidant properties; however it has yielded mixed results in human populations (Agarwal and Behari, 2007). Dietary fibre may confer some protection from MeHg exposure by reducing absorption (Rowland et al., 1986). A reduction of MeHg absorption was the hypothesized mechanism for the reduced MeHg body burdens detected in individuals from the Amazon basin who have high tropical fruit consumption (Passos et al., 2007).

The present study focuses on the interaction between MeHg body burden from fish intake and the consumption of other dietary items (fruits, vegetables, coffee, teas, whole grains, and eggs) or nutrients (sum of antioxidant or fibre intake). The objectives of this study were to 1) determine if there are populations within Costa Rica that are at risk for high Hg exposure from fish consumption, 2) gather detailed dietary information to determine the daily MeHg intake for individuals in one of these populations, and 3) explore if there are any nutrient-MeHg interactions. The daily MeHg intake for people residing in different locations in Costa Rica was determined by estimating each location’s general fish consumption and determining the average local fish-Hg concentrations. In one of these
populations, we conducted a cross-sectional epidemiology study to determine daily MeHg intake using personal dietary questionnaires and hair sampling. Hair sampling has been validated as a good biomarker for MeHg exposure in fish eating populations (Passos et al., 2008), and is often the preferred method of assessing a person’s exposure as it is non-invasive, does not require immediate processing, and represents a longer exposure period than blood (Cernichiari et al., 1995). This study was a part of a larger international capacity building project, including the establishment of an Hg analysis centre and a local student training program.

5.2 Methodology

5.2.1 Sampling Costa Rican fish

Information was gathered about potential natural and anthropogenic point sources of Hg or MeHg production into the environment in Costa Rica, which included volcanic activity, gold mining operations, hydroelectric reservoirs, wetlands, industrial activity, and deforestation. Information was gathered from local experts and maps at the Universidad Nacional, as well as publically available information at the Costa Rican Ministry of the Environment (MINAE, 2010). Based on this information, a total of 14 communities were selected for further risk assessment investigation; Miramar, Limon, Cachí, Costa de Pajaros, San Luis, Paquera, Mal Pais, Cahuita, Puntarenas, Los Chilies, Heredia, Colorado, Guatuso and Caño Negro (Fig. 5.1). At each community we recorded the general fish consumption habits based on local interviews, and categorized it as 1) “sustenance fish consumption”
Figure 5.1  Map of Costa Rica indicating the 14 locations of the risk assessment study. (Map adapted from Window Adventure, 2010)

Locations:
1) Miramar
2) Limon
3) Cachi
4) Costa de Pajaros
5) San Luis
6) Paquera
7) Mal Pais
8) Cahuita
9) Puntarenas
10) Los Chilies
11) Heredia
12) Colorado
13) Guatuso
14) Caño Negro
(the majority of dietary protein comes from locally caught fish, 5+ meals/wk), 2) “moderate fish consumption” (2-4 fish meals a week), or 3) “light fish consumption” (0-1 fish meals a week). To approximate the level of MeHg intake resulting from this fish consumption, we also assessed the Hg concentrations of various fish eaten in each location. Locally consumed fish were purchased from fishermen, community freezers, or markets. When possible, whole fish were photographed, weighed, measured for total length (mouth to tail), and body length (back of head to base of tail). A 10 g sample of each fish was taken from the dorsal anterior section of each fish, or from the prepared fish fillet or steak. Canned tuna was purchased at local markets. All samples were divided into 2 pieces and placed in separate plastic bags, labelled, and kept on ice for up to 12 hours before they were stored at -20°C. One set of samples was kept in the Atmospheric Chemistry Laboratory (LAQAT) of the Universidad Nacional in Heredia, Costa Rica, and the other set of samples was shipped to the Centre for Advanced Research in Environmental Genomics (CAREG) at the University of Ottawa, Canada. Fish species were identified based on their common name, and later confirmed by biologists at the Universidad Nacional and reference taxonomical identification book (Bussing, 2002).

5.2.2 Determination of fish-Hg concentrations

Total Hg (THg) was quantified at CAREG by thermal decomposition followed by dual step gold amalgamation and detection by cold vapour atomic absorption spectrophotometry using a SP-3D mercury analyzer (Nippon Instruments, Japan) as described previously (Al-Reasi et al., 2007). Approximately 10 mg of unprocessed sample was placed in a ceramic boat
covered with layers of additive M (sodium carbonate and calcium hydroxide) and additive B (aluminum oxide), and placed into the thermal decomposition chamber for analysis. Quality control of THg recovery was assessed every 5 samples with standard reference material (DORM-2, dogfish muscle, National Research Council, Ottawa, Canada), which remained within the certified values of 4.64 ± 0.26 mg THg/kg. The detection limit for THg was 0.01 ng.

5.2.3 MeHg exposure risk assessment of Costa Rican populations

A general risk assessment for each location was estimated based on the locally reported level of fish consumption and the average fish-Hg concentration of the local diet according to previously described methods (WHO, 2008). The locally reported level of fish consumption was entered into calculations as: “sustenance fish consumption” (5 meals a week), “moderate fish consumption” (3 fish meals a week), or “light fish consumption” (1 fish meal a week). The average fish-Hg concentration of the local diet was calculated based on the reported types of fish consumed and the fish-Hg concentrations of the fish. An estimated daily MeHg intake per person was calculated for each location.

\[
\text{Daily MeHg intake per person in location A} = \\
[Average \text{ dietary fish-Hg concentration in location A (µg/g)} \times \]
\[
\text{Standard fish portion (150 g)} \times \]
\[
\text{Frequency fish consumption in location A (1 or 3 or 5 meals/week)}] \]
\[
/\text{Standard body weight (60 kg)} \times \]
\[
7 \text{ (day/week)}
\]
The daily MeHg intake per person in each location was compared to the pTDI of 0.2 µg/KgBW/d (Health Canada, 2007a). The population of each location was obtained from the Costa Rica Government’s 2000 census (INEC, 2000). The community with the highest estimated general daily MeHg intake and greatest population at risk was selected for further investigation with a cross-sectional dietary survey study.

5.2.4 Dietary survey development

Prior to the initiation of the full epidemiological study, a pilot project was performed to optimize the dietary survey for cultural and linguistic correctness, train a local student research assistant, and test the data analysis methodology. Full ethical approval was obtained from the Centre for Ethical Conduct at the Universidad Nacional (CECUNA). The initial dietary survey comprised 15 questions with a mixture of continuous and ordinal answers. Similar questions were asked in different manners to determine inconsistencies and potential wording issues. The pilot project ran for 2 months involving approximately 45 participants from LAQAT and adjacent laboratories at the Universidad Nacional. Interviews were conducted with 10 randomly selected participants after the completion of the survey to assess the readability of the survey and to gather any suggestions for improvement. The dietary survey information was coded, entered into a database, and statistical analysis was conducted. The main dietary survey was then developed based on the results of our pilot project.
5.2.5 Dietary assessment of participants

Each participant’s average dietary habits over the past 12 months were assessed using a food frequency questionnaire (FFQ), which included an informed consent form, and detachable identification page (Appendix A). The FFQ collected detailed information from several food categories, as well as smoking and alcohol consumption, vitamin/supplement intake, hair treatments, and metal amalgams. Frequency of consumption of each food item was defined as the number of times a food item or “portion” was included in a meal. The 3 page dietary survey contained 19 questions with blank spaces to provide continuous answers and blank spaces to report items not listed in each category. Participant’s total fibre intake was calculated as the sum of the frequency of consumption of individual fibre-rich items or “portions”: whole grains, fruits, and vegetables. Similarly, participant’s total antioxidant intake was calculated as the sum of the frequency of consumption of individual antioxidant-rich items or “portions”: fruits, fruit juices, vegetables, coffee, and teas. Upon collection of the completed dietary questionnaire and hair sample from each participant, the hair sample was coded along with the identification page, which removed from the remaining survey to ensure participant confidentiality.

5.2.6 Study design and population

A cross-sectional dietary study was undertaken among the student and staff population of the Universidad Nacional in Heredia, Costa Rica (Fig. 5.1). Recruitment was conducted using convenience sampling, a strategy demonstrated to accurately represent
the underlying population (Kelly et al., 2002), which in our study, was the general university population. We were invited to present the study during staff meetings and classroom lessons, where the purpose of the study was explained and individuals were invited to participate on a voluntary basis. Informed consent was obtained from each participant, along with a completed food survey and a hair sample. Approximately 300 individuals were invited to participate, which resulted in 278 study participants, achieving 93% participation rate. Reasons for exclusion were inability or refusal to provide a hair sample, inadequate time to fill out the survey, or unknown. Completed surveys with a paired hair sample were used in the analysis (n = 268). Volunteers who provided a hair sample were informed of their Hg-hair concentration as being either above or below the 2 µg/g recommended limit. At this time they also received information about the benefits of including fish in their diet and an outline of WHO recommendations for healthy fish choices (WHO, 2008). Full ethical approval was obtained from CECUNA.

5.2.7 Calculation of daily MeHg intake

The daily MeHg intake was calculated for each participant using their reported consumption of each fish species and the average mercury concentration measured for that fish species. Values were calculated according to gender-specific fish meal portion (females = 125 g, males = 190 g) and body weight (females = 53 kg, males = 60 kg) according to previously published data (Passos et al., 2008).

\[
\text{Daily MeHg intake} = [\text{Average fish A concentration (µg/g)} \times \ldots]
\]
Gender-specific fish meal portion size (125 or 190 g) x 
Frequency fish A consumption (meals/day)] + 
[Average fish B concentration (µg/g) x 
Gender specific fish meal portion size (125 or 190 g) x 
Frequency fish B consumption (meals/day)] + ... + 
[Average fish Z concentration (µg/g) x 
Gender specific fish meal portion size (125 or 190 g) x 
Frequency fish Z consumption (meals/day)] 
/ Gender specific body weight (53 or 60 kg)

5.2.8 Hair sampling and analysis

Hair mercury levels has been validated as a good biomarker for MeHg exposure in fish eating populations (Passos et al., 2008), and is often the preferred method of assessing a person’s exposure as it is non-invasive, does not require immediate processing, and represents a longer exposure period than blood (Cernichiari et al., 1995). At the time of survey collection, a small bundle of hair strands measuring 1-2 mm in diameter was isolated from basal occipital region of each participant and cut at the root with scissors. The cut ends of the hair strands were placed in an 8 cm X 8 cm piece of paper, secured with a staple, labelled with the code from its paired dietary survey, and placed in a small plastic bag. THg was quantified in the same manner as the fish samples described in section 5.2.2.
5.2.9 Statistical analysis

Descriptive statistics were used to characterize the study population demographics, consumption patterns, Hg-Hair concentrations, and daily MeHg intake. Homogeneity and distribution were assessed and parametric or non-parametric analyses were applied accordingly. Correlation analyses were used to detect relationships between Hg-Hair concentrations and fish consumption or daily MeHg intake. Intergroup comparisons were made based on dietary habits, socio-economic criteria, and smoking or drinking habits. The influence of various dietary variables on the relationship between Hg-hair (dependant variable) and fish consumption (independent variable) were assessed with step-wise linear regression models. Continuous variables were used except for gender, home location, and vitamin/supplement use. Participants reporting the use of permanent hair treatments (n = 2) were excluded from analysis as this may interfere with Hg-hair (Dakeishi et al., 2005). Other potential covariates, such as alcohol consumption, smoking, gender and age, were included in the models. Analysis of covariance (ANCOVA) was used to test interactions. Results were considered statistically significant when p < 0.05. JMP 8.0 (SAS Institute, USA) was used to conduct all analyses.

5.3 Results

5.3.1 Fish-Hg concentrations

The Hg concentrations were determined for 151 fish samples representing 25 species collected from 14 different locations across Costa Rica (Table 5.1). The mean Hg
Table 5.1 Mercury concentrations of fish species collected from locations within Costa Rica.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Location</th>
<th>Hg (ng/g)</th>
<th>n</th>
<th>Feeding habits</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bagre (Sciades seemanni)</td>
<td>Colorado</td>
<td>90.9 ± 19.5</td>
<td>3</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td>Chulin (Rhamdia rogersi)</td>
<td>Guatuso</td>
<td>149.9 ± 65.5</td>
<td>2</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td>Cichlid (Amphiliophus sp.)</td>
<td>Los Chiles</td>
<td>332.4 ± 116.6</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Common snook (Centropomus undecimalis)</td>
<td>Costa de Pajaros</td>
<td>68.5 ± 20.4</td>
<td>3</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td></td>
<td>Los Chiles</td>
<td>86.3 ± 11.7</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Conger (Conger sp.)</td>
<td>Heredia</td>
<td>206.7 ± 70.6</td>
<td>3</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Croaker (Cynoscion sp.)</td>
<td>Costa de Pajaros</td>
<td>56.7 ± 17.2</td>
<td>3</td>
<td>Carnivorous</td>
</tr>
<tr>
<td></td>
<td>Paquera</td>
<td>157.7 ± 85.4</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Dolphinfish (Coryphaena sp.)</td>
<td>Heredia</td>
<td>354.0 ± 2.6</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td></td>
<td>Puntarenas</td>
<td>310.2 ± 17.3</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Flying fish (Cheilopogon sp.)</td>
<td>Colorado</td>
<td>175.2 ± 34.9</td>
<td>5</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td>Grouper (Epinephelus labriformis)</td>
<td>Mal Pais</td>
<td>43.1 ± 0.6</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Grunt (Pomadasys sp.)</td>
<td>Colorado</td>
<td>73.5 ± 10.8</td>
<td>4</td>
<td>Carnivorous</td>
</tr>
<tr>
<td></td>
<td>Rio San Carlos</td>
<td>46.6 ± 21.4</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Guapote (Parachromis sp.)</td>
<td>Guatuso</td>
<td>222.2 ± 142.1</td>
<td>2</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td></td>
<td>San Luis (farmed)</td>
<td>253.5 ± 44.9</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Los Chiles</td>
<td>132.5 ± 7.4</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Los Chiles (farmed)</td>
<td>59.2 ± 0.2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Jackfish (Seriola zonata)</td>
<td>Cahuita</td>
<td>2268.0 na</td>
<td>1</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Marlin (Tetrapturus sp.)</td>
<td>Puntarenas</td>
<td>204.2 ± 4.1</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Sabalo (Brycon behreae)</td>
<td>Guatuso</td>
<td>461.6 ± 64.6</td>
<td>3</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Fish species</td>
<td>Location</td>
<td>Hg (ng/g)</td>
<td>n</td>
<td>Feeding habits</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>----------------</td>
<td>-------------</td>
<td>----</td>
<td>----------------</td>
</tr>
<tr>
<td>Sailfish (Istrophorus platypterus)</td>
<td>Puntarenas</td>
<td>1146.8 ± 50.9</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Salmon (Oncorhynchus sp.)</td>
<td>Heredia</td>
<td>25.5 ± 1.9</td>
<td>2</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td>Sardines (family Clupeidae)</td>
<td>Guatuso</td>
<td>41.6 ± 8.5</td>
<td>8</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td>Sawfish (Pristis sp.)</td>
<td>Colorado</td>
<td>330.1 ± 127.7</td>
<td>4</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Small tailed shark (Carcharhinus porosus)</td>
<td>Heredia</td>
<td>660.9 ± 234.7</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Snapper (Lutjanus sp.)</td>
<td>Cahuita</td>
<td>194.6 ± 4.9</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td></td>
<td>Limon</td>
<td>58.0 ± 4.6</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mal Pais</td>
<td>59.0 ± 6.1</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Puntarenas</td>
<td>60.0 ± 20.5</td>
<td>4</td>
<td></td>
</tr>
<tr>
<td>Swordfish (Xiphias gladius)</td>
<td>Puntarenas</td>
<td>600.9 ± 78.0</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Tilapia (Oreochromis sp.)</td>
<td>Los Chiles (farmed)</td>
<td>44.1 ± 3.2</td>
<td>3</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td></td>
<td>Miramar (farmed)</td>
<td>51.6 ± 9.2</td>
<td>6</td>
<td></td>
</tr>
<tr>
<td>Tropical gar (Atractosteus tropicus)</td>
<td>Caño Negro</td>
<td>347.3 ± 11.4</td>
<td>5</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Trout (Oncorhynchus mykiss)</td>
<td>Rio Volio (farmed)</td>
<td>134.6 ± 4.2</td>
<td>5</td>
<td>Non-carnivorous</td>
</tr>
<tr>
<td>Tuna - Canned (Thunnus sp.)</td>
<td>Heredia</td>
<td>283.1 ± 59.6</td>
<td>16</td>
<td>Carnivorous</td>
</tr>
<tr>
<td>Tuna - Fresh (Thunnus sp.)</td>
<td>Puntarenas</td>
<td>312.3 ± 3.8</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
<tr>
<td></td>
<td>Heredia</td>
<td>1047.2 ± 47.7</td>
<td>2</td>
<td>Carnivorous</td>
</tr>
</tbody>
</table>
concentration of the 25 species was 293.4 ng/g. Of these, the 8 non-carnivorous species had an average Hg concentration of 109.7 ng/g, whereas the 17 carnivorous species had an average Hg concentration of 400.6 ng/g. The Hg concentrations of the non-carnivorous fish ranged from 25.5 ng/g (salmon from Heredia) to 253.5 ng/g (guapote farmed in Lake Arenal). The Hg concentrations of the carnivorous fish ranged from 43.1 ng/g (grouper from Mal Pais) to 2268.0 ng/g (jackfish from Cahuita). Several species were sampled from multiple locations including croaker, dolphinfish, grunt, snapper, tuna, common snook, guapote, and tilapia, none of which had a significant interlocation variation of Hg concentrations (Kruskal-Wallis, P < 0.05). The Hg concentration of farmed guapote was significantly lower than wild guapote, which were both sampled from Los Chiles (Mann-Whitney U; P < 0.05).

The concentration of THg detected in canned tuna purchased from Heredia markets was highly variable with a 10-fold difference between the lowest and highest concentration (Fig. 5.2). The canned tuna were coded prior to analysis and represented the most commonly purchased brands; Bumblebee™, Gomes de Costa Rica™, Calvo™, Atún Azul™, Real Mar™, Herdez™, Tesoro Mar™, Sabemas™, Sardimar™, and Splash™. The samples of canned tuna originated from tuna packaged in Costa Rica, Mexico, Thailand, Ecuador, and El Salvador. Seven of the 30 cans of tuna, from 5 different brands exceeded the 500 ng/g legal sales limit in Canada for canned tuna (Health Canada, 2007a). Brands # 9 and 10 were removed from analysis as they only had one collection replicate. The average THg concentration was not statistical different between the brands of canned tuna or the
Mercury concentrations in 10 brands of canned tuna purchased from Heredia, Costa Rica, from June 2008 to April 2009 presented as A) Hg concentration per can ($n = 32$), and B) average mercury concentrations in each brand of canned tuna ($n = 1-4$). The dashed lines represent the legal sales limit for canned tuna in Canada (Health Canada, 2007a).
country of origin of the tuna. The canned tuna purchased in Costa Rica did not have labels indicating the type of tuna, such as “albacore” or “light tuna”, types typically known to have lower Hg concentrations.

5.3.2 MeHg risk assessment in Costa Rican populations

The estimated total daily intake of MeHg from fish consumption per person in each of the 14 locations was calculated based on local fish-Hg concentrations and level of fish consumption (Fig. 5.3). Of these, 5 had a daily intake of MeHg above the Health Canada recommended limit of 0.2 µg/KgBW/d: Los Chiles, Caño Negro, Heredia, Colorado, and Guatuso.

Los Chiles residents had an estimated MeHg exposure 0.22 µg/KgBW/d. This is a small town (population of 10,000) in Northern Costa Rica at the boarder of Nicaragua. The fish from this location primarily come from the surrounding river systems that link the community to Lake Nicaragua and the San Juan River. This community was of interest since it is downstream of operational chlor-alkali plants in Lake Nicaragua, a known source of Hg contamination leading to MeHg contamination in fish. Guapote, common snook and cichlid are the main species of fish caught here. There is also a large tilapia farm here but most of the stock is reserved for export. The guapote caught here had the lowest levels in the country (Table 5.1). The calculated daily intake of MeHg was primarily due to the consumption of cichlid fish and a high reliance on fish as part of the diet.
Figure 5.3 Estimated daily MeHg intake from fish consumption by individuals residing in different locations in Costa Rica (Number) corresponds to map location in Figure 5.1. The dashed line represents the recommended provisional tolerable daily intake for MeHg (Health Canada, 2007a).
Colorado residents had an estimated MeHg exposure 0.31 µg/KgBW/d. This is a small fishing community (population of 3,482) in the Gulf of Nicoya on the Pacific side of Costa Rica. This population had a high reported fish consumption, which included sawfish, a high trophic-level ocean fish, contributing to their high estimated MeHg exposure.

Guatuso residents also had an estimated MeHg exposure 0.31 µg/KgBW/d. This is a small Makela Indigenous reserve (population of 1,115) in northern Costa Rica close to Tenorio volcano. Guatuso was of interest due to the population’s high reliance on locally caught fish and close proximity to volcanic activity.

Caño Negro residents had an estimated MeHg exposure 0.54 µg/KgBW/d. This is a remote fishing community (population 1,594) situated within the country’s largest wetland and east of an active volcanic ridge including volcano Arenal, Tenorio, Miravalles, and Rincon de la Vieja. This community was of interest due to its proximity to active volcanoes, favourable environmental conditions for Hg methylation and subsequent aquatic food chain contamination, and the population’s high fish consumption. Indeed, the fish-Hg levels here were high in guapote and tropical gar compared to other locations and fish species. The combination of high fish consumption and high fish-Hg concentrations resulted in the highest calculated daily MeHg intake.

Heredia residents had an estimated MeHg exposure 0.27 µg/KgBW/d. This is a small city (population of 100,000) located 50 km North of San Jose, the capital of Costa Rica. Heredia, San Jose, Alajuela and a few other adjacent cities are located in Costa Rica’s central
valley, which hosts 50% (2.2 million) of the country's inhabitants. It was surprising to observe a high daily MeHg intake in Heredia as it was sampled as a control population. Although Heredia was indicated as having a generalized “medium” (3 meals/wk) fish consumption, the preference for canned tuna and the high concentrations of Hg in canned tuna contributed to a high daily MeHg intake. Due to the high daily MeHg and the large population that may be impacted, Heredia was selected as the location to conduct the dietary survey study.

5.3.3 Dietary Survey

A total of 278 individual volunteers enrolled in the study. We collected hair samples from 275 individuals, dietary surveys from 271 individuals, resulting in 268 completed hair-survey pairs. The complete 268 hair-surveys were used for further analysis.

The socio-demographic information for the male and female study participants were characterized (Table 5.2). The average age was 21.9 years with a range of 15 to 52 years. Seventy six percent (76%) of participants reported alcohol consumption within the past week, and 18% of participants reported smoking tobacco within the past week. Forty one percent (41%) of participants reported living in Heredia, while the remainder commuted from adjacent locations such as the capital San Jose (15%), Alajuela (13%) and other locations (31%).

The participant hair-Hg concentrations followed a normal distribution with a tapering right skew (Fig. 5.4). One individual was more than 3 standard deviations above the
Table 5.2  Socio-demographic characterization of the sampled population.

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Women</th>
<th></th>
<th>Men</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N</td>
<td>%</td>
<td>N</td>
<td>%</td>
</tr>
<tr>
<td><strong>Age categories</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>15 to 19</td>
<td>67</td>
<td>46.9</td>
<td>56</td>
<td>45.2</td>
</tr>
<tr>
<td>20 to 24</td>
<td>49</td>
<td>34.3</td>
<td>47</td>
<td>37.9</td>
</tr>
<tr>
<td>25 to 29</td>
<td>11</td>
<td>7.7</td>
<td>11</td>
<td>8.9</td>
</tr>
<tr>
<td>≥ 30</td>
<td>16</td>
<td>11.2</td>
<td>10</td>
<td>8.1</td>
</tr>
<tr>
<td>Total</td>
<td>143</td>
<td>100</td>
<td>124</td>
<td>100</td>
</tr>
<tr>
<td><strong>Current alcohol consumer</strong></td>
<td>113</td>
<td>79.0</td>
<td>90</td>
<td>72.6</td>
</tr>
<tr>
<td><strong>Current smoker</strong></td>
<td>22</td>
<td>15.4</td>
<td>25</td>
<td>20.2</td>
</tr>
<tr>
<td><strong>Home location</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>San Jose</td>
<td>23</td>
<td>16.1</td>
<td>16</td>
<td>12.9</td>
</tr>
<tr>
<td>Heredia</td>
<td>54</td>
<td>37.8</td>
<td>55</td>
<td>44.4</td>
</tr>
<tr>
<td>Alajuela</td>
<td>21</td>
<td>14.7</td>
<td>14</td>
<td>11.3</td>
</tr>
<tr>
<td>Other</td>
<td>45</td>
<td>31.5</td>
<td>39</td>
<td>31.5</td>
</tr>
</tbody>
</table>
Figure 5.4 Distribution of mercury concentrations in hair (µg/g) of participants (n = 269).
mean with a hair-Hg level of 10.9 µg/g, which was considered a statistical outlier and removed from further analysis. The average hair Hg concentration was $1.8 \pm 0.1$ µg/g (median: 1.6 µg/g, min: 0.1 µg/g, max: 9.6 µg/g). There was no difference in hair-Hg concentrations between men and women. The WHO’s recommended limit for maternal hair, 2 µg/g, was exceeded by 40% of the female participants.

The participants reported eating a variety of items from multiple categories of dietary nutrients (Table 5.3). In this study, 267 of the participants (99%) reported eating fish within the past year. Of these people, 260 consumed at least one meal containing carnivorous fish (97%) and 188 consumed at least one meal containing non-carnivorous fish (70%). The average Hg-fish concentration and average frequency of fish consumption was calculated for each fish species (Table 5.4). The average Hg concentrations in fish were calculated from fish purchased in Heredia or fish purchased at fish farms that supplied Heredia supermarkets. On average participants consumed $1.3 \pm 0.1$ fish meals a week, which varied greatly depending on the type of fish (Table 5.4). The most commonly consumed fish was canned tuna ($0.9 \pm 0.1$ meals/week), with croaker ($0.4 \pm 0.03$ meals/week) being a distant second choice, both carnivorous fish types. Ceviche, the third most commonly reported fish consumed, is a popular marinated raw fish salad typically made from croaker or dolphinfish. The average concentration of ceviche was calculated as an average of both these species. Tilapia ($49.1 \pm 9.2$ ng/g) and snapper ($59.4 \pm 6.1$ ng/g) had the lowest levels of Hg, and were more frequently consumed compared to tuna fillets ($1047.0 \pm 39.0$ ng/g) and shark ($660.9 \pm 87.2$ ng/g), which had the highest levels of Hg.
Table 5.3 Types of food items included in the food frequency survey.

<table>
<thead>
<tr>
<th>Food groups</th>
<th>Food item specified in food frequency survey</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish</td>
<td>Croaker, tuna, tilapia, trout, salmon, snapper, marlin, dolphinfish, swordfish, shark</td>
</tr>
<tr>
<td>Fruit</td>
<td>Banana, berries, pineapple, citrus, mango, cas, guava, melon, papaya, apple, pear, grapes</td>
</tr>
<tr>
<td>Vegetables</td>
<td>Dark green vegetables: beans, lettuce, etc; Red/Orange vegetables: carrots, tomatoes, etc.</td>
</tr>
<tr>
<td>Medicinal teas</td>
<td>Camomile, linden, juanilama, mint, good herb, noni, ginkgo biloba, basil, rosemary</td>
</tr>
<tr>
<td>Beverages</td>
<td>Coffee, black tea, green tea, herbal tea</td>
</tr>
<tr>
<td>Grains</td>
<td>Rice, whole wheat bread, pasta, cereals, oats, flour products</td>
</tr>
<tr>
<td>Nuts</td>
<td>Peanuts, walnuts, almonds</td>
</tr>
<tr>
<td>Eggs</td>
<td>Chicken eggs</td>
</tr>
</tbody>
</table>
Table 5.4 Frequency (portions/week) and mercury concentrations (ng/g) of specific types of fish consumed by participants in Heredia.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Frequency of consumption (meals/wk)</th>
<th>Hg concentration (ng/g)</th>
<th>Feeding habits</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SEM</td>
<td>Range</td>
<td>Mean ± SEM</td>
</tr>
<tr>
<td>Canned tuna (Thunnus sp.)</td>
<td>0.9 ± 0.05</td>
<td>0 - 7.0</td>
<td>296.4 ± 59.6</td>
</tr>
<tr>
<td>Croaker (Cynoscion sp.)</td>
<td>0.4 ± 0.03</td>
<td>0 - 5.0</td>
<td>97.1 ± 37.8</td>
</tr>
<tr>
<td>Ceviche (various)*</td>
<td>0.3 ± 0.03</td>
<td>0 - 5.0</td>
<td>215.1 ± 153.1</td>
</tr>
<tr>
<td>Tilapia (Oreochromis sp.)</td>
<td>0.3 ± 0.03</td>
<td>0 - 5.0</td>
<td>49.1 ± 9.2</td>
</tr>
<tr>
<td>Small tailed shark (Carcharhinus porosus)</td>
<td>0.1 ± 0.02</td>
<td>0 - 2.0</td>
<td>660.9 ± 87.2</td>
</tr>
<tr>
<td>Trout (Oncorhynchus sp.)</td>
<td>0.1 ± 0.02</td>
<td>0 - 1.5</td>
<td>135 ± 4.2</td>
</tr>
<tr>
<td>Tuna fillets (Thunnus sp.)</td>
<td>0.1 ± 0.01</td>
<td>0 - 2.0</td>
<td>1047.0 ± 39.0</td>
</tr>
<tr>
<td>Snapper (Lutjanus sp.)</td>
<td>0.1 ± 0.01</td>
<td>0 - 2.0</td>
<td>59.4 ± 6.1</td>
</tr>
<tr>
<td>Dolphinfish (Coryphaena sp.)</td>
<td>0.1 ± 0.01</td>
<td>0 - 2.0</td>
<td>333.1 ± 10.2</td>
</tr>
<tr>
<td>Marlin (Tetrapturus sp.)</td>
<td>0.1 ± 0.01</td>
<td>0 - 1.0</td>
<td>204.2 ± 3.3</td>
</tr>
<tr>
<td>Swordfish (Xiphias gladius)</td>
<td>0.0 ± 0.00</td>
<td>0 - 0.5</td>
<td>600.1 ± 78.0</td>
</tr>
<tr>
<td>Guapote (Parachromis dovii)</td>
<td>0.0 ± 0.00</td>
<td>0 - 3.0</td>
<td>175.4 ± 27.9</td>
</tr>
<tr>
<td>Others</td>
<td>0.0 ± 0.01</td>
<td>0 - 2.0</td>
<td>-</td>
</tr>
<tr>
<td>Total non-carnivorous fish</td>
<td>1.7 ± 0.04</td>
<td>0 - 9</td>
<td>528.3 ± 214.7</td>
</tr>
<tr>
<td>Total carnivorous fish</td>
<td>0.5 ± 0.08</td>
<td>0 - 8</td>
<td>280.8 ± 115.5</td>
</tr>
<tr>
<td>Total fish</td>
<td>1.3 ± 0.06</td>
<td>0 - 6.0</td>
<td>321.4 ± 86.9</td>
</tr>
</tbody>
</table>

* Ceviche is typically made from dolphinfish or croaker. The mean Hg concentration was calculated as the average of these two species.
The daily MeHg intake from fish consumption was calculated for each participant (Fig. 5.5). The average daily MeHg intake of all participants was 0.19 ± 0.01. The males in the study had an average daily MeHg intake of 0.21 ± 0.02 µg/KgBW/d, and 8.9% of male participants exceeded the pTDI for MeHg of 0.47 µg/KgBW/d (Health Canada, 2007a). On the other hand, the female participants had an average daily MeHg intake of 0.18 ± 0.01 µg/KgBW/d, and 34% of these people exceeded the Canadian pTDI of 0.2 µg/KgBW/d (Health Canada, 2007a). Although there was no statistically significant difference between the daily MeHg intake of males and females, the lower pTDI for women of reproductive age indicated a greater proportion of females to be at risk.

In order to elucidate the most significant sources of MeHg exposure, the average daily MeHg intake of participants resulting from each type of fish species consumed was determined (Fig. 5.6). In this study, the most significant source of MeHg dietary exposure was from the consumption of canned tuna, which had an average participant exposure of 0.08 ± 0.01 µg/KgBW/d. The maximum calculated Hg exposure was 0.95 µg/KgBW/d for one individual, primarily due to their reported tuna consumption. The ceviche, shark, and tuna filets were the next most significant source of MeHg exposure, each contributing an average of 0.03 µg/KgBW/d. The consumption of carnivorous fish provided 90% of the total MeHg intake. No associations were observed between Hg exposure or fish consumption with age, gender, home location, smoking or drinking status.

Hair-Hg was positively correlated to fish consumption (Rho = 0.254, P < 0.001) and the calculated participant daily MeHg intake (Rho = 0.225, P < 0.001) (Fig. 5.7). Since we
Figure 5.5  Distribution of the participants’ daily MeHg intake (µg/KgBW/d) from fish consumption.
Figure 5.6  Daily MeHg intake of study participants from each fish species, total fish, carnivorous fish, and non-carnivorous fish.
Figure 5.7  Relationship between participants’ Hg hair concentration (µg/g) and A) fish consumption \( (y = 0.3x + 1.5, \text{Rho} = 0.25, n= 266, p < 0.001) \) or B) calculated Hg intake from fish consumption \( (y = 1.2x + 1.6, \text{Rho} = 0.23, n= 266, p < 0.001) \)
found a slightly better association with fish consumption as a predictor of hair-Hg, we used this parameter for further investigations. Partial correlation analysis of the relationship between hair-Hg and fish consumption of each type of fish were conducted to elucidate their respective contribution to exposure (Table 5.5). As expected, carnivorous fish had a stronger correlation (Rho = 0.240, P < 0.001) than non-carnivorous fish (Rho = 0.188, P = 0.002). Canned tuna was the form of fish consumption with the strongest correlation (Rho = 0.171, P = 0.005). Interestingly, this was followed by tilapia (Rho = 0.159, P = 0.009) and trout (Rho = 0.155, P = 0.011), both non-carnivorous fish with relatively low Hg concentrations and a high frequency of consumption (Table 5.4), which may have been more accurately reported compared to less common fish eaten only occasionally.

The dietary antioxidant intake of participants was determined from their reported intake of fruits, fruit juices, vegetables, coffees and teas (Table 5.6). There were a high percentage of participants who ate at least one item of fruit (99.6%), vegetable (98.2%), coffee (73.8%), or tea (77.1%) within the past week. The most commonly consumed fruit was a citrus juice made from lemons or oranges (93.0%), followed by bananas (90.4%) and berries (89.3%). There was a large variation of the consumption of fruit, vegetables, coffee and teas (portions/week) between participants, which represent the majority of antioxidant intake for individuals (mean ± SD: 35.2 ± 22.6, range: 2.5-182, median: 30 portions/week).

Participants were then divided into two groups based on their overall antioxidant intake at the median (30 portions/week). Regression lines were plotted for “low” antioxidant consumers (<30 antioxidant portions/week; n = 136) and “high” antioxidant
Table 5.5  Relationship between frequency of fish species consumption (meals/week) and hair Hg (ng/g).

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Feeding habits</th>
<th>Spearman’s rho</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Canned tuna (Thunnus sp.)</td>
<td>Carnivorous</td>
<td>0.171*</td>
<td>0.005</td>
</tr>
<tr>
<td>Croaker (Cynoscion sp.)</td>
<td>Carnivorous</td>
<td>0.126*</td>
<td>0.040</td>
</tr>
<tr>
<td>Ceviche (various)</td>
<td></td>
<td>0.108</td>
<td>0.078</td>
</tr>
<tr>
<td>Tilapia (Oreochromis sp.)</td>
<td>Non-carnivorous</td>
<td>0.159*</td>
<td>0.009</td>
</tr>
<tr>
<td>Small tailed shark (C. porosus)</td>
<td>Carnivorous</td>
<td>0.085</td>
<td>0.166</td>
</tr>
<tr>
<td>Trout/salmon (Oncorhynchus sp.)</td>
<td>Non-carnivorous</td>
<td>0.155*</td>
<td>0.011</td>
</tr>
<tr>
<td>Yellowfin tuna (Thunnus sp.)</td>
<td>Carnivorous</td>
<td>0.050</td>
<td>0.415</td>
</tr>
<tr>
<td>Snapper (Lutjanus sp.)</td>
<td>Carnivorous</td>
<td>0.149*</td>
<td>0.015</td>
</tr>
<tr>
<td>Dolphinfish (Coryphaena sp.)</td>
<td>Carnivorous</td>
<td>0.149*</td>
<td>0.014</td>
</tr>
<tr>
<td>Marlin (Tetrapturus sp.)</td>
<td>Carnivorous</td>
<td>0.024</td>
<td>0.694</td>
</tr>
<tr>
<td>Swordfish (Xiphias gladius)</td>
<td>Carnivorous</td>
<td>0.024</td>
<td>0.691</td>
</tr>
<tr>
<td>Guapote (Parachromis dovii)</td>
<td>Non-carnivorous</td>
<td>0.013</td>
<td>0.836</td>
</tr>
<tr>
<td>Others</td>
<td></td>
<td>0.061</td>
<td>0.318</td>
</tr>
<tr>
<td>Total non-carnivorous fish</td>
<td>Non-carnivorous</td>
<td>0.188*</td>
<td>0.002</td>
</tr>
<tr>
<td>Total carnivorous fish</td>
<td>Carnivorous</td>
<td>0.240*</td>
<td>0.000</td>
</tr>
<tr>
<td>Total fish</td>
<td></td>
<td>0.261*</td>
<td>0.000</td>
</tr>
</tbody>
</table>

* Spearman’s rho correlation coefficients statistically significant at $p < 0.05$
Table 5.6  Descriptive statistics of dietary antioxidant items consumed (portions/week)

<table>
<thead>
<tr>
<th>Antioxidant item</th>
<th>Mean ± SEM (portions/week)</th>
<th>Range</th>
<th>Median</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fruit</td>
<td>9.9 ± 0.5</td>
<td>0-49</td>
<td>7</td>
</tr>
<tr>
<td>Bananas</td>
<td>2.5 ± 0.2</td>
<td>0-14</td>
<td>1</td>
</tr>
<tr>
<td>Berries</td>
<td>1.5 ± 0.2</td>
<td>0-35</td>
<td>0.8</td>
</tr>
<tr>
<td>Pineapple</td>
<td>1.6 ± 0.2</td>
<td>0-21</td>
<td>1</td>
</tr>
<tr>
<td>Citrus</td>
<td>3.7 ± 0.3</td>
<td>0-35</td>
<td>2</td>
</tr>
<tr>
<td>Mangos</td>
<td>1.7 ± 0.2</td>
<td>0-21</td>
<td>0.8</td>
</tr>
<tr>
<td>Others</td>
<td>2.8 ± 0.2</td>
<td>0-32</td>
<td>1.3</td>
</tr>
<tr>
<td><strong>Vegetables</strong></td>
<td>7.4 ± 0.4</td>
<td>0-35</td>
<td>7</td>
</tr>
<tr>
<td>Dark greens</td>
<td>3.3 ± 0.2</td>
<td>0-28</td>
<td>2</td>
</tr>
<tr>
<td>Reds &amp; Oranges</td>
<td>7.1 ± 0.4</td>
<td>0-35</td>
<td>7</td>
</tr>
<tr>
<td><strong>Beverages</strong></td>
<td>13.2 ± 0.8</td>
<td>0-77</td>
<td>10.3</td>
</tr>
<tr>
<td>Coffee</td>
<td>7.7 ± 0.5</td>
<td>0-56</td>
<td>5</td>
</tr>
<tr>
<td>Black tea</td>
<td>1.6 ± 0.2</td>
<td>0-28</td>
<td>0</td>
</tr>
<tr>
<td>Green tea</td>
<td>1.6 ± 0.2</td>
<td>0-21</td>
<td>0.3</td>
</tr>
<tr>
<td>Herbal/medicinal tea</td>
<td>2.0 ± 0.3</td>
<td>0-49</td>
<td>0.3</td>
</tr>
<tr>
<td><strong>Total antioxidant items</strong></td>
<td>35.3 ± 1.4</td>
<td>2.5-182</td>
<td>29.5</td>
</tr>
</tbody>
</table>
consumers (≥ 30 antioxidant portions/week; n = 130). The dietary habits and socio-demographics we characterized for low and high antioxidant consumer (Table 5.7). Total antioxidant consumption was correlated to fish consumption (Rho = 0.192; p = 0.002) and age (Rho = 0.138; p = 0.024). There were differences of total antioxidant consumption across smoking (Mann-Whitney U; p = 0.038) and drinking status (Mann-Whitney U; p = 0.025). There was no relation observed for total antioxidant intake and gender or reported home location.

Fish consumption and total antioxidant intake entered significantly into multiple regression analysis for hair-Hg of participants (Table 5.8). When genders were entered independently into the model only the males reached statistical significance. This interaction was present regardless of socio-demographic factors. When the individual antioxidant items (fruit, vegetables, coffee, teas) were entered individually no significant interaction was detected. Other dietary parameters, including intake of total fibre, eggs, or nuts, also did not result in a significant interaction with hair Hg concentrations. The inverse relationship of total antioxidant intake remained significant when the sum of carnivorous and non-carnivorous fish consumption were entered separately. Similarly, a significant inverse relationship of total antioxidant intake was present when the calculated daily MeHg intake of participants was used instead of fish consumption in the model. There was no relationship between hair-Hg concentrations with number of dental amalgams or the reported number of illnesses (data not shown). The interaction of antioxidant intake on the relationship between fish consumption and resulting hair-Hg concentrations in participants
Table 5.7  Characteristics of study participants identified as low antioxidant consumers (<30 antioxidant portions/week; \( n = 136 \)) or high antioxidant consumers (≥ 30 antioxidant portions/week; \( n = 130 \)). Different letters indicate significant differences between high and low antioxidant consumers at \( p < 0.05 \).

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>High antioxidant consumers</th>
<th>Low antioxidant consumers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mean ± SEM</td>
<td>mean (%)</td>
</tr>
<tr>
<td><strong>Home location</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>San Jose</td>
<td>17 (13.0)</td>
<td>21 (15.6)</td>
</tr>
<tr>
<td>Heredia</td>
<td>58 (44.3)</td>
<td>54 (40.0)</td>
</tr>
<tr>
<td>Alajuela</td>
<td>14 (10.7)</td>
<td>21 (15.6)</td>
</tr>
<tr>
<td>Other</td>
<td>42 (32.1)</td>
<td>41 (30.4)</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>22.9 ± 0.68</td>
<td>21.1 ± 0.47</td>
</tr>
<tr>
<td><strong>Gender</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female</td>
<td>73 (55.7)</td>
<td>70 (51.1)</td>
</tr>
<tr>
<td>Male</td>
<td>58 (44.3)</td>
<td>67 (48.9)</td>
</tr>
<tr>
<td><strong>Current alcohol consumers</strong></td>
<td>104 (79.4)(^a)</td>
<td>98 (71.5)(^b)</td>
</tr>
<tr>
<td><strong>Current smokers</strong></td>
<td>29 (22.3)(^a)</td>
<td>20 (14.6)(^b)</td>
</tr>
<tr>
<td><strong>Hg in hair (µg/g)</strong></td>
<td>1.77 ± 0.09</td>
<td>1.98 ± 0.12</td>
</tr>
<tr>
<td><strong>Hg exposure</strong></td>
<td>0.25 ± 0.02(^a)</td>
<td>0.15 ± 0.01(^b)</td>
</tr>
<tr>
<td><strong>Fish consumption</strong></td>
<td>1.53 ± 0.1(^a)</td>
<td>1.15 ± 0.07(^b)</td>
</tr>
<tr>
<td>Carnivorous</td>
<td>1.99 ± 0.14(^a)</td>
<td>1.39 ± 0.08(^b)</td>
</tr>
<tr>
<td>Non-Carnivorous</td>
<td>0.57 ± 0.08(^a)</td>
<td>0.37 ± 0.04(^b)</td>
</tr>
</tbody>
</table>
Table 5.8  Multiple regression analysis of the interaction of antioxidant intake (portions/week) on the relationship between hair-Hg concentration (ng/g) and fish consumption.

<table>
<thead>
<tr>
<th>Outcome</th>
<th>n</th>
<th>Regression estimates</th>
<th>Model $R^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair Hg (ng/g)</td>
<td></td>
<td>Fish consumption</td>
<td>Total antioxidant intake</td>
</tr>
<tr>
<td>Women</td>
<td>142</td>
<td>315 ($P = 0.003$)</td>
<td>-287 ($P = 0.134$)</td>
</tr>
<tr>
<td>Men</td>
<td>121</td>
<td>356 ($P &lt; 0.001$)</td>
<td>-534 ($P = 0.005$)</td>
</tr>
<tr>
<td>Total</td>
<td>263</td>
<td>335 ($P &lt; 0.001$)</td>
<td>-382 ($P = 0.005$)</td>
</tr>
</tbody>
</table>
demonstrated that for low antioxidant consumers every fish meal per week resulted in an elevation of 1.82 µg/g hair-Hg, whereas for high antioxidant consumers every fish meal per week resulted in an elevation of 1.67 µg/g hair-Hg (Fig. 5.8). For example, if an individual consumed on average 3 fish meals a week, if they are also a high antioxidant consumer their hair-Hg would be approximately 2 µg/g, alternatively, if they were also a low antioxidant consumer their hair-Hg would be approximately 2.6 µg/g. This 30% difference results in the low antioxidant consumer exceeding the pTDI for woman of reproductive age, whereas the high antioxidant consumer does not. We choose to use fish consumption as an indicator of MeHg exposure because it resulted in a better correlation to hair-Hg than the calculated daily MeHg intake. Interestingly, the high antioxidant consumers had slightly higher reported fish consumption and daily MeHg intake, yet the interaction of antioxidants on lowering Hair-Hg persisted.

5.4 Discussion

In this study we determined fish-Hg concentrations in various locations across Costa Rica to explore possible point sources of Hg contamination, estimated the general daily MeHg intake for individuals at each location to identify a population that may be at risk of excessive MeHg exposure, and conducted an epidemiological study within this identified population to determine the dietary factors that contribute to and interact with Hg body burden.
Figure 5.8  Relationship between participants’ Hg hair concentration (µg/g) and fish consumption (meals/week) in participants with A) a low antioxidant intake (<30 antioxidant portions/week) \( y = 0.4x + 1.4, R^2 = 0.13, P < 0.00 \), and B) a high antioxidant intake (≥ 30 antioxidant portions/week) \( y = 0.2x + 1.4, R^2 = 0.02, P = 0.25 \).
We collected the species of fish primarily eaten in each location visited, and although the common species displayed location-dependant trends of Hg concentration, they were not significantly different. This lack of detection may have been due to a limited sample set at the various locations. For example, snapper collected from three geographically distinct locations had a consistent fish-Hg concentration of approximately 60 ng/g \((n = 12)\), whereas the concentration of snapper collected from Cahuita was more than 3 times higher \((n = 2)\), although this difference did not reach statistical significance. Interestingly, Cahuita was also the location of the collection of the fish with the highest detected level of Hg, 2268 ng/g in Jackfish. Unfortunately this particular fish species of fish was only available at this Caribbean location and in a single specimen, making interlocation and intraspecies comparisons impossible. Interestingly, the coral and sediment samples collected in Costa Rica had higher Hg concentrations on the Caribbean coast than the Pacific coast (Guzman and Garcia, 2002). Fish-Hg concentrations in farmed guapote from Los Chiles were significantly lower than wild guapote from Los Chiles, which may be due to their diet, water-Hg levels, or different age at time of capture. As expected, carnivorous fish generally had higher levels of Hg compared to non-carnivorous fish.

Canada’s Health Products and Food Branch and the USA’s Food and Drug Administration both have extensive fish-Hg monitoring systems enabling the comparison of Costa Rican fish-Hg levels by fish type (Table 5.9). On average the fish-Hg concentrations we determined in our Costa Rican study were 1.5 higher than the same fish types in Canada or USA. This was not consistent throughout all species as the ratio of Hg-fish between Costa
Table 5.9. Comparison of Hg concentrations (µg/g) in fish species in Costa Rica, USA, and Canada.

<table>
<thead>
<tr>
<th>Fish species</th>
<th>Costa Rica Hg (µg/g) ± SEM</th>
<th>USA(^a) Hg (µg/g) ± SD</th>
<th>CR:USA</th>
<th>Canada(^b) Hg (µg/g)</th>
<th>CR:Can</th>
</tr>
</thead>
<tbody>
<tr>
<td>Croaker (Cynoscion sp.)</td>
<td>0.107 ± 0.051</td>
<td>0.287 ± 0.069</td>
<td>0.4</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Dolphinfish (Coryphaena sp.)</td>
<td>0.332 ± 0.010</td>
<td>na</td>
<td>na</td>
<td>0.220 ± na</td>
<td>1.5</td>
</tr>
<tr>
<td>Grouper (Epinephelus labriformis)</td>
<td>0.043 ± 0.001</td>
<td>0.465 ± 0.293</td>
<td>0.1</td>
<td>0.325 ± na</td>
<td>0.1</td>
</tr>
<tr>
<td>Marlin (Tetrapturus sp.)</td>
<td>0.204 ± 0.004</td>
<td>0.485 ± 0.237</td>
<td>0.4</td>
<td>0.915 ± na</td>
<td>0.2</td>
</tr>
<tr>
<td>Salmon (Oncorhynchus sp.)</td>
<td>0.026 ± 0.002</td>
<td>0.014 ± 0.041</td>
<td>1.8</td>
<td>0.030 ± na</td>
<td>0.9</td>
</tr>
<tr>
<td>Sardines (family Clupeidae)</td>
<td>0.042 ± 0.008</td>
<td>0.016 ± 0.007</td>
<td>2.6</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Shark (Family Carcharhinae)</td>
<td>0.661 ± 0.235</td>
<td>0.988 ± 0.631</td>
<td>0.7</td>
<td>1.283 ± na</td>
<td>0.5</td>
</tr>
<tr>
<td>Snapper (Lutjanus sp.)</td>
<td>0.093 ± 0.009</td>
<td>0.189 ± 0.274</td>
<td>0.5</td>
<td>0.070 ± na</td>
<td>1.3</td>
</tr>
<tr>
<td>Swordfish (Xiphias gladius)</td>
<td>0.601 ± 0.078</td>
<td>0.976 ± 0.510</td>
<td>0.6</td>
<td>1.118 ± na</td>
<td>0.5</td>
</tr>
<tr>
<td>Tilapia (Oreochromis sp.)</td>
<td>0.048 ± 0.006</td>
<td>0.010 ± 0.023</td>
<td>4.8</td>
<td>na</td>
<td>na</td>
</tr>
<tr>
<td>Trout (Oncorhynchus mykiss)</td>
<td>0.135 ± 0.004</td>
<td>0.072 ± 0.143</td>
<td>1.9</td>
<td>0.040 ± na</td>
<td>3.4</td>
</tr>
<tr>
<td>Tuna - Canned (Thunnus sp.)</td>
<td>0.283 ± 0.060</td>
<td>0.118 ± 0.119</td>
<td>2.4</td>
<td>0.050 ± na</td>
<td>5.7</td>
</tr>
<tr>
<td>Tuna - Fresh (Thunnus sp.)</td>
<td>0.680 ± 0.026</td>
<td>0.383 ± 0.269</td>
<td>1.8</td>
<td>0.613 ± na</td>
<td>1.1</td>
</tr>
</tbody>
</table>

\(^a\) (USFDA, 2009)
\(^b\) (Health Canada, 2007a)
Rica and Canada ranged from 0.1 to 5.7, and between Costa Rica and USA ranged from 0.1-4.8. The largest difference between fish-Hg occurred in the canned tuna between Costa Rica and Canada. The average concentration we measured in Costa Rican canned tuna was 0.283 ± 0.060 µg/g Hg, which is 5.7 times higher than the reported Canadian canned tuna. Furthermore, in Canada and other countries, canned tuna must be labelled with the specific species of tuna. For example, Canadian sold yellowfin or skipjack tuna has lower Hg concentrations than larger tuna species such as albacore (Health Canada, 2007a). None of the canned tuna in Costa Rica had such labels. Dolphinfish, sardines, tilapia, trout, and tuna (fresh) all had higher fish-Hg concentrations in Costa Rica compared to Canada or the USA. On the other hand, swordfish from Canada and the USA had 0.5 and 0.6 times the amount of Hg than Costa Rican brands, respectively. Croaker, grouper, marlin and shark all had lower fish-Hg concentrations in Costa Rica compared to Canada or the USA.

Canada’s Food Inspection Agency prohibits the sale of fish that have Hg concentrations above 0.5 µg/g (Health Canada, 2007a). Six specialty fish are exempt from this restriction and instead are subjected to a 1.0 µg/g limit as they are considered less commonly consumed, which include orange roughy, marlin, escolar, tuna steaks, shark and swordfish (Health Canada, 2007a). Costa Rica does not have established Hg concentration limits for fish sales. In our study, jackfish from Cahuita, sailfish from Puntarenas, swordfish from Puntarenas, as well as tuna steaks and canned tuna from Heredia exceeded these sales limits. For example, 23% of the canned tuna purchased from the Heredia supermarket exceeded the 0.5 µg/g Hg limit. It is clear that a regulatory body should be established in
Costa Rica to set a legal sales limit for Hg concentrations in fish and canned tuna, as well as a monitoring and enforcement program.

Fish is an important source of nutrients and macronutrients known to be beneficial to neurodevelopment (Daniels et al., 2004). Fish is rich in protein, low in saturated fats, and high in polyunsaturated fats such as omega 3 and omega 6. They are also an important source of selenium and vitamin E, which may modulate MeHg toxicity (Beyrouty and Chan, 2006). The concept that nutrients from fish provide health benefits that can modulate or ameliorate MeHg toxicity has created confusion within the public on the role of fish as part of a healthy diet (Mozaffarian and Rimm, 2006). The benefits of fish consumption need to be a part of publically promoted food guidelines, which include information on healthy fish choices that maximize nutrient intake and minimize MeHg exposure. Furthermore, the benefits and harm from fish consumption have been identified as an important confounder to correct in MeHg epidemiology studies (Choi et al., 2008), which may have contributed to differential outcomes between two large developmental MeHg exposure cohort studies (Myers and Davidson, 1998).

An estimated daily MeHg intake was calculated for individuals residing within 14 different locations throughout Costa Rica. Based on general fish consumption by persons in each location and average local fish-Hg concentrations, five locations were identified as having an estimated daily MeHg intake exceeding the MeHg pTDI for women of childbearing age of 0.2 µg/KgBW/d; Los Chiles, Heredia, Colorado, Guatuso, and Caño Negro. With the exception of Heredia, these locations were remote communities with less than 10,000
residents and their high daily MeHg intake was primarily due to high fish consumption. Heredia on the other hand, is a city centre representing a modern urban population with moderate fish consumption. The majority of the fish consumed in Heredia was from canned tuna, which also had higher Hg concentrations than Canada or the USA. The previously conducted MeHg-dietary nutrient interaction epidemiology studies have focused on small, rural, fish eating populations (Passos et al., 2003; Passos et al., 2007), therefore it was unknown if the observed trends would be applicable to other populations. Thus we choose Heredia as the location of our Hg exposure epidemiology study. Future studies should conduct further assessments of the remaining 4 locations, particularly Caño Negro, which had the highest estimated daily MeHg intake.

The estimated daily MeHg intake was later confirmed in Heredia using a cross-sectional epidemiological study that collected detailed dietary information and measured hair-Hg as an indicator of Hg body burden. By calculating each participant’s MeHg exposure from their reported fish consumption and fish-Hg concentrations, we were able to confirm that individuals within Heredia, particularly women, are at risk of excessive MeHg exposure. In Heredia, the average daily MeHg intake was 0.19 ± 0.01 µg/KgBW/d, and 34% of female participants exceeded the recommended pTDI guideline of 0.2 µg/KgBW/d. Using hair-Hg as a biomarker for MeHg exposure and body burden resulted in similar findings, where 40% of females in our study exceeded the recommended maternal hair limit of 2 µg/g.

It is interesting to note that the estimated daily MeHg intake in Heredia for the general risk assessment study and dietary survey were 0.27 µg/KgBW/d and 0.19 ± 0.1
μg/KgBW/d, respectively. Although these values were calculated using generalized information about local fish eating habits and average fish-Hg concentrations, or detailed dietary survey information, there was only a 30% difference between these two values.

The results of the dietary survey suggested the ability of dietary antioxidant intake to reduce Hg body burden from fish consumption. This reduction was present regardless of smoking or drinking habits, and in all age categories. However, when males and females were entered separately into multiple regression modeling this antioxidant interaction was only apparent for the male participants. It is possible that food recall bias for our FFQ was more pronounced with female participants, which has been observed in past studies. Interestingly, this reduction was present despite the higher MeHg intake and fish consumption of high antioxidant consumers. Although antioxidant intake was correlated to smoking and drinking status, these potential confounding variables did not enter significantly into regression modeling, indicating they are not true interactions. These results suggest that the public promotion of a balanced diet that includes fish and antioxidant rich items such as fruits, vegetables, teas and coffees, may provide a method to reduce the risk associated from Hg exposure without altering fish consumption.

A plausible hypothesis for the mechanism of the observed reduction in Hg body burden from antioxidant consumption is the prevention of MeHg demethylation within cells due to the improved oxidative status of individuals who consume an antioxidant-rich diet. Evidence to support this hypothesis comes from the observations that an antioxidant-rich diet provides a plethora of health benefits including improved oxidative stress protection.
within bodily tissues (Mier-Cabrera et al., 2009). These benefits are extended to the CNS as dietary antioxidants have been associated with preventative of the neurodegeneration occurring from the ROS accumulation due to aging (Lau et al., 2007), and Alzheimer’s and Parkinson’s diseases (Uttara et al., 2009). It has also been noted that an increase in ROS within the cellular environment increases the rate of MeHg demethylation into inorganic Hg (Shapiro and Chan, 2008). Inorganic Hg is the form of Hg that accumulates within brain cells (Friberg and Mottet, 1989), and elicits the most potent neurotoxicity once within the CNS (Basu et al., 2008). By preventing cellular MeHg demethylation and Hg accumulation, a greater proportion of MeHg may enter the enterohepatic cycle to be excreted from the body. Furthermore, an improved cellular oxidative state would result in a great proportion of the intracellular glutathione to be in its reduced form as GSH. Since GSH-MeHg conjugates promote cellular export of MeHg, it is possible that an improved cellular oxidative state may once again facilitate MeHg to enter the enterohepatic cycle for excretion.

In addition to these proposed processes occurring within the cellular environment, it is possible that the antioxidant phytochemicals derived from the diet are binding with MeHg in the gut and reducing MeHg absorption. Indeed MeHg is known bind to plant based tannins as part of industrial remediation (Gaballah and Kilbertus, 1998) and have been suggested as a significant interaction in various MeHg exposure animal model studies (Chapman and Chan, 2000). For example, raw garlic was able to reduce Hg accumulation in
rats chronically exposed to MeHg (Cha, 1987). A toxicodynamic study of MeHg and plant-based antioxidants would need to be completed to further assess these hypotheses.

A similar interaction of dietary nutrients and MeHg was found in a remote Brazilian Amazon population, which was attributed to the consumption of tropical fruits (Passos et al., 2003; Passos et al., 2007). In this case it was suggested that the dietary fibre present in the fruit was interfering with MeHg absorption in the intestinal track, which reduced the Hg body burden. However, in our study it was only the sum total of antioxidant consumption that demonstrated the interaction between fish consumption and Hg body burden, whereas the sum total of fibre did not demonstrate this interaction. It is interesting to note that in the Brazilian study the strongest interaction was seen with orange consumption (Passos et al., 2007), a fruit well known for its high ascorbic acid (vitamin C) content and potent antioxidant activities. Plant derived compounds have also demonstrated MeHg toxicity protection in cell culture, for example, γ-linolic acid was able to reduce DNA damage in lymphocyte cell cultures (Bala et al., 1993). This interaction has been observed in vivo also, where medicinal plant extracts successfully ameliorated or attenuated MeHg induced neurotoxicity, including Halimeda increassata (Linares et al., 2004), Polygala paniculata (Farina et al., 2005) and Cipura paludosa (Lucena et al., 2007). Furthermore, we observed the modulation of developmental MeHg toxicity with Rhododendron tomentosum ssp. subarcticum (Black et al., 2011 in press).
5.5 Conclusions

In summary, we measured fish-Hg concentrations that exceeded Canada’s legal sales limit in several Costa Rican fish species including canned tuna. The estimated daily MeHg intake from fish consumption also exceeded Canada’s pTDI in five of the 14 locations assessed across Costa Rica. Heredia, a small, modern city, was one of these locations and was selected for further assessment of MeHg exposure with a dietary epidemiology study. Here we found a significant positive correlation between Hg-hair and fish consumption, and between Hg-hair and calculated daily MeHg intake. This relationship was influenced by the antioxidant intake of the individual, such that increased antioxidant intake reduced Hg-hair regardless of fish intake.

Together these results indicate that several populations within Costa Rica, including those residing in rural traditional communities and urban cities, are exposed to mercury levels that may increase the risk of adverse health effects. Further assessments of these populations and a fish-Hg sales regulatory body or public health intervention may be required to prevent MeHg-associated adverse health outcomes. Additionally, it is possible that the risk of excess Hg exposure may be mitigated through the promotion of a well balanced diet that includes fish and antioxidant-rich items such as fruits, vegetables, teas and coffee.
CHAPTER SIX

General Discussion and Conclusions

6.1 Thesis summary

This doctoral thesis research explores the interaction between dietary antioxidants and MeHg. The first objective was to find an antioxidant source that was culturally appropriate and readily available for the Inuit of Canada, a population known to have elevated MeHg exposure (Chapter Two). To achieve this we conducted an ethnobotanical study and collected various medicinal plants for laboratory analyses. We determined that Northern Labrador Tea (*Rhododendron tomentosum* ssp. *subarcticum*) was a commonly used medicinal plant and herbal tea by multiple First Nations groups, including the Inuit from Nunavut, Canada. Furthermore, extracts of *R. tomentosum*, referred to as “Tea” herein, had potent antioxidant properties. In preparation for a toxicological study on the interactions of Tea on MeHg induced neurotoxicity, the phytochemical composition and seasonal variation of Tea was characterized. The major constituents of Tea were (+)-catechin, quercetin-pentoside and quercetin-3-O-galactoside, were found at their highest concentrations in the fall, which corresponded to peak antioxidant activity and the traditional time of plant harvesting by Inuit. All three of these phytochemicals are well known antioxidants, and quercetin is commercially available as a natural health product.
The second objective was to determine how Tea would modify the developmental, behavioural, and biochemical outcomes associated with developmental MeHg exposure (Chapter Three). To achieve this we used a perinatal exposure study where pregnant and lactating rats were dosed with MeHg and/or Tea. We observed that developmental MeHg exposure was associated with decreased survival and growth, increased lipid peroxidation and brain NMDA receptor levels, decreased grip strength, hyperactivity and loss of central aversion in open field testing, and delayed acoustic startle response time. On the other hand, MeHg had no effect on acoustic startle amplitude and prepulse inhibition, timing of eye and ear opening, and on GSH concentration in serum, cerebellum and cerebrum. These findings were generally consistent with the published literature on the effects of MeHg (Johansson et al., 2007; Pelletier et al., 2009; Beyrouty and Chan 2006; Farina et al., 2004), and to our knowledge, was the first time NMDA-R binding perturbation has been reported following developmental MeHg exposure. Although Tea had almost no effect on these endpoints, co-administration with MeHg appeared to alter MeHg pharmacokinetics: THg and MeHg concentrations in blood were generally higher with an elevated demethylation rate. Despite the increased blood mercury levels in the MeHg + Tea exposed animals, it did not contribute to an increase in adverse effects compared to MeHg exposure alone. However, Tea co-exposure abolished MeHg effects on brain NMDA-R levels and altered MeHg effects on lipid peroxidation. This may be due to the similar Hg concentrations in the pups’ CNS tissue, despite the elevated Hg blood concentrations. Finally, although exposure to MeHg or Tea administered alone had no effect on the timing of eye opening, co-exposure
resulted in precocious eye opening, an observation also reported following MeHg co-exposure with antioxidants (Beyrouty and Chan, 2006) and contaminants (Vitalone et al., 2008), an observation with unknown consequences.

To attempt to conceptualize this interaction between the antioxidant-rich Tea and developmental MeHg exposure, we hypothesized that the antiseptic properties of this medicinal plant may have disrupted the gut flora of the rats, resulting in decreased gut demethylation rates and decreased excretion of MeHg. Indeed the ethnobotanical portion of our study suggested the antiseptic use of this medicinal plant (Table 2.1), and previous animal MeHg dosing studies have noted the elevation of Hg body burden following antibiotic administration (Rowland et al., 1984). In the present study, this elevated Hg concentration was only apparent in the blood, perhaps due to the lag time of brain deposition and the young age of our pups. Despite this elevated Hg blood concentration compared to pups dosed with MeHg alone, co-exposed pups had drastically improved NMDA-R levels in the CNS. It is therefore tempting to postulate that the Tea was able to directly or indirectly reduce MeHg-induced oxidative stress. Indeed, we observed that serum lipid peroxidation levels in Tea co-exposed pups were at control levels by PND 21.

The third objective was to determine the molecular targets of developmental MeHg exposure and MeHg + Tea co-exposure, and to link these targets with our previous results (Chapter Four). We used a subset of samples from the perinatal exposure to conduct a microarray experiment. Interestingly, the molecular targets of MeHg did not appear to be associated with oxidative stress defense or glutamate balance, two of the best
characterized mechanisms of neurotoxicity induction. Instead, MeHg administered alone was found to cause transcriptional perturbations related to translation, cell cycle, cell division, and DNA metabolism. Although Tea alone was associated with translation, its co-exposure with MeHg was not associated with any functional GO categories. Similar findings were detected with the KEGG pathway analysis. Since MeHg exposure began on GD 0, five weeks prior to the collection of our PND 14 samples to conduct this experiment, we hypothesize that the transcriptional response captured reflected the systemic toxicity of the pups rather than the immediate adaptive response. Interestingly, using a less stringent cut off criteria, both MeHg and MeHg + Tea treatments were associated with functional GO categories related to blood circulation, wounding, and blood pressure. Corroborating these results, multiple studies have reported the damaging effect of MeHg exposure on cardiovascular functions in humans (Stern, 2005; Virtanen et al., 2007; Fillion et al., 2006; Grandjean et al., 2004), on microvascular endothelial cells (Hirooka et al., 2009), and the blood-brain barrier (G.Pelletier, unpublished data).

Furthermore, we found that developmental MeHg exposure induced the down-regulation of 2 related genes within the NR4A nuclear transcription gene family, *Nr4a2* and *Nr4a3*. These genes code for early response genes following neuronal NMDA-R stimulation (Zetterstrom et al., 1996; Dragunow et al., 1996), suggested to be crucial for the development of learning and memory. The observation of the down-regulation *Nr4a2* was corroborated with the detection of the down-regulation of Nurr1, the protein product of
Both the \textit{Nr4a2} transcription and Nurr1 protein product were returned to control levels following Tea co-exposure.

Although completely speculative at this point, if we link the behavioural, molecular, and brain receptor results together we may gather a more complete understanding of the impact of developmental MeHg exposure and Tea interactions. In our study, MeHg exposure was associated with hyperactivity and a trend of thigmotaxis loss, which have been previously observed following the administration of NMDA-R antagonist (Koros et al., 2007; Adamec et al., 1999; Plaznik et al., 1994). This suggests that MeHg can interfere with NMDA-R signalling. This idea is further supported as we detected a significant elevation of NMDA-Rs, perhaps in response to a loss of excitatory signal. Our study could not determine if this increased NMDA-R binding was due to a larger population of NMDA-R expressing cells or an increased cell surface concentration of NMDA-Rs. The cell surface concentration of NMDA-Rs is regulated by poorly characterized pathways, but considering their crucial role in development, it is reasonable to assume that a reduction in NMDA-R signalling would result in an elevation of cell surface NMDA-Rs. The hypothesized loss of NMDA-R signalling is further supported with our gene expression and immunoblot results. NMDA-R stimulation is associated with the up-regulation of early response genes such as the Nr4a gene family. In our study, we found a significant down-regulation of Nr4a2 and Nr4a3, suggesting that the NMDA-R is not adequately stimulated. The interference of NMDA-R signalling corroborates the widely accepted role of NMDA-Rs and glutamate excitotoxicity in MeHg neurotoxicity. Here we suggest that MeHg exposure resulted in a loss of NMDA-R signalling and a
subsequent up-regulation of cell surface receptors. Perhaps most interesting was the rescue of NMDA-R levels and NMDA-R induced Nr4a gene/protein expression with Tea co-exposure. This suggested that these endpoints may be perturbed by MeHg-induced oxidative stress, and that dietary antioxidants may relieve such toxic endpoints.

The laboratory animal model study allowed us to assess the interactions between the antioxidant-rich Tea and MeHg toxicity as measured with developmental milestones, behavior, biochemistry and molecular biology, as well as to investigate new targets of toxicity. However there exists scant information about the interactions of dietary antioxidants on MeHg in human populations.

Our last objective was to determine if there were significant interactions of dietary antioxidants and MeHg body burden in humans, which we approached using a cross-sectional epidemiology study conducted in Costa Rica (Chapter Five). By conducting a preliminary risk assessment survey in 14 different locations across Costa Rica, we identified Heredia as having the greatest risk from MeHg exposure based on their population size, fish concentrations and consumption habits. In Heredia, 40% of female participants had hair-Hg concentration that exceeded the recommended limit of 2 µg/g where there may be an increased risk of adverse effects following in utero MeHg exposure (WHO, 2003).

Corroborating this observation, we calculated each participant’s daily MeHg intake from fish consumption and found that 34% of woman participants exceeded the pTDI of 0.2 µg/KgBW/d (Health Canada, 2007). This high level of MeHg exposure from fish was primarily attributed to the consumption of canned tuna, which had Hg concentrations exceeding
Canadian legal sales restrictions of 500 ng/g in 23% of the samples. Canned tuna was the most commonly consumed fish, with an average weekly consumption of 0.9 portions and a reported range of 0 to 7 portions. There are no legal sales restrictions for fish-Hg levels or fish consumption guidelines in Costa Rica. Using the collected dietary information from each participant we calculated their total antioxidant consumption, including portions of fruits, fruit juices, vegetables, coffee, and teas. By dividing the participants at the median antioxidant consumption, we categorized participants as high (≥30 portion/week) or low (<30 items/week) antioxidant consumers. Interestingly, we detected that dietary antioxidants could significantly reduce the Hg body burden resulting from fish consumption. To our knowledge, this study was the first to identify the reduction of MeHg body burden resulting from fish consumption by dietary antioxidants in an urban population.

6.2 Contributions to the understanding of MeHg developmental toxicity and nutrient interactions

Just as ecotoxicology is a multidisciplinary field of science, this doctoral research provided contributions in the areas of ethnobotany, plant pharmacology, classical toxicology, molecular biology, toxicogenomics, and epidemiology. The results from this doctoral thesis provide evidence of the ability of antioxidants to interact with MeHg at various endpoints and in both an animal model and a human population. Specifically, the data generated from our rat model experiment supports the existing literature on the developmental, behavioral, and transcriptional response outcomes following developmental MeHg exposure, and provides new evidence for the mechanisms of this
neurotoxicity. The mitigation of the MeHg-induced elevation of NMDA-Rs binding levels and molecular perturbations of translation, cell cycle, cell division, and DNA metabolism by the supplementation with Tea did not translate into a detectable improvement of behavioral or developmental outcomes in our rat model. This suggests a complex relationship between phenotypic toxicity and the underlying mechanisms. It is possible that although the Tea did make improvements to the toxic outcomes of MeHg exposure, the high dose of MeHg was such that these benefits were largely masked by a general systemic toxicity.

Interestingly, our results also indicated that Tea supplementation may have altered MeHg toxicokinetics, as we observed slightly higher tissue Hg concentrations following MeHg + Tea co-exposure compared to MeHg exposure alone. Although the mechanism remains elusive, it is tempting to speculate that antibacterial properties of the plant caused a disruption in the demethylation processes occurring by the maternal intestinal gut flora, resulting in a decrease in fecal elimination, and elevated MeHg transfer into fetal blood and tissues. Indeed, the ethnobotanical usage of this plant suggests its antiseptic qualities (Chapter Two, Table 2.1) and other species within the Rhododendron genus have reported bactericidal properties (Innocenti et al., 2010).

In contrast, our epidemiological study demonstrated the ability of a diverse antioxidant-rich diet to reduce the MeHg body burden resulting from fish consumption. This finding has corroborated previous studies conducted in rural fish-eating populations (Passos et al., 2003; Passos et al., 2007) and may provide further evidence for an alternative approach to reducing the risk associated with MeHg exposure, without compromising the
intake of nutrients from fish consumption. Here we have hypothesized that the antioxidant-rich diet helped improve the oxidative status of the cellular environment, which minimized demethylation processes and reduced the level of Hg accumulation within tissues. In order to consolidate our results regarding the interaction of dietary nutrients on MeHg toxicokinetics a model was developed (Fig. 6.1).

In light of the results from the animal model experiment and epidemiological study, these interactions appear to be dependent on the source of dietary antioxidant (single medicinal plant extract or complete plant-based diet) and/or the duration of exposure (during development or life-long). It is also possible that these nutrient-MeHg interactions manifest in a species dependant manner (humans or rats) and/or according to the dose of MeHg exposure (clinical or subclinical levels).

Together these results demonstrate the ability of dietary antioxidants to interact on MeHg-induced neurotoxicity, toxicokinetics and body burden. This interaction has implications for the accurate MeHg exposure risk assessment and may provide a method for risk reduction.
Figure 6.1  Schematic depiction of the hypothesized sites of dietary nutrient interaction with MeHg toxicokinetics. “Tea” refers to the *R. tomentosum* leaf extract used in the animal dosing study in Chapter 3-4, whereas “Antioxidants” refers to compounds that may derive from the “Tea” or a diverse diet as discussed in the human population study in Chapter 5. Refer to text for further explanation.
6.3 Study limitations and future directions

The second and third objectives of this doctoral thesis were to determine how Tea would modify the developmental, behavioural, biochemical, and transcriptional outcomes associated with developmental MeHg exposure, which were achieved by conducting a rat perinatal MeHg exposure experiment. The observation of elevated Hg residues in pup tissue following developmental co-exposure to MeHg and Tea was not expected and may have been due to several different situations. On one hand it is possible that the Tea supplementation allowed for the survival of more contaminated pups, which in turn elevated the average Hg tissue concentrations for this group. Alternatively, it is possible that Tea caused a disruption of the maternal gut flora, which reduced the demethylation and the excretion of MeHg. To elucidate between these two hypotheses, the Hg concentrations in maternal blood, feces, or in deceased pup tissues could have been measured, samples that were not collected in this study. Furthermore, since we found a variety of effects of Tea co-exposure with respect to lipid peroxidation levels in the blood and brain and different time points, we hypothesized that the concentrations of plant phytochemicals and MeHg varied in the various compartments over the course of exposure. Future perinatal exposure studies should include toxicokinetic and phytochemistry components to determine how co-exposure scenarios alter the distribution of Hg and phytochemicals throughout maternal and pup tissue compartments, with particular attention to the alteration of MeHg demethylation rates in the gut. It may also be of interest to conduct an animal model perinatal MeHg exposure study with a proportion of the animals also receiving a
standardized diet that includes a variety of dietary antioxidants. This would allow a better understanding of the impact of antioxidants on MeHg metabolism within the system, and avoid potential antiseptic actions on the gut flora.

Ideally toxicological studies contain multiple biologically relevant dosing levels to establish a contaminant dose-response curve. In our study we had a single MeHg exposure level (2.0 mg/KgBW/d) that resulted in a high pup mortality rate despite a previous study conducted at Health Canada (data not published) suggested this was a suitable dose. Since our study, our group at Health Canada has adopted a 1.2 mg/KgBW/d standard dose. Future rat perinatal studies should be conducted at multiple lower doses such as 0.02 and 0.2 mg/KgBW/d to generate dose-response data for chronic developmental MeHg exposures, particularly for the detection of subtle molecular changes with microarray analyses. However due to the factorial design of mixture studies, the number of treatment groups dramatically increases with every additional compound, an issue further compounded by the labor intensive nature of perinatal studies. Future studies that include a lower Hg dose would be particularly useful for toxicogenomic and mechanistic studies for the detection of targeted brain pathways. In our case, we chose to avoid the possibility that a low MeHg dose such as 0.02 mg/kg may not produce a reliable or appreciable phenotypic toxicity, which would have restricted our ability to detect interactions with Tea. Despite these limitations our rat perinatal experiment produced excellent proof-of-concept results that antioxidant phytochemicals can interact with MeHg toxicity.
Although the agreement between Nr4a2 mRNA and its protein product, Nurr1, validated our microarray results, ideally RT-PCR should have been conducted to confirm microarray data. Based on our results we would have chosen these 5 genes for RT-PCR; NR4a2, NR4a3, Ppp1r7, Ppp2r5c, and Nmral1.

The final objective of this doctoral thesis was to determine if there were significant interactions of dietary antioxidants and MeHg, which we approached using a cross-sectional epidemiology study conducted in Costa Rica. The limitations of our study include those common to epidemiology studies. There is always a trade off between the amount of data collected from each individual and the sample size of a study. Here we opted to utilize a relatively short food survey (19 questions in 3 pages) while obtaining between 250-300 participants to gather focused dietary information with an adequate detection power. We used convenience sampling within the university campus for our study, a validated method for representing the underlying population (Kelly et al., 2002), and as a result our participants did not represent the general public demographics of Heredia in age or socio-economic status. Despite this limitation we decided to use this study approach to target the average maternal age of the first pregnancy in Costa Rica, an important vulnerable subpopulation. We achieved a 93% participation rate, with well distributed diet and demographic characteristics. The use of food frequency questionnaires is a well accepted method for estimating long-term nutrient intake but because of its retrospective nature it can be prone to recall bias (Margetts and Nelson, 2000). There is also always a level of biological and technical inaccuracy when measuring fish-Hg concentrations. Biological
inaccuracy was minimized within this study by sampling as many fish as possible, which was sometimes impossible due to logistical difficulties associated with rural locations, rotating fishing bans issued by the Costa Rican government, and limited collection resources. To minimize technical inaccuracies, hair and fish samples were analyzed for Hg levels in the CAREG Hg laboratory, a nationally certified Hg laboratory for quality control. Any inaccuracies of the reported fish consumption or fish-Hg levels would be compounded when calculating individual daily MeHg intakes, which may have been a factor contributing to the stronger correlation between hair-Hg and fish consumption than hair-Hg and daily MeHg intake. Lastly, the use of biomarkers instead of direct measurement always has inherent obstacles. Here we used hair-Hg concentrations to predict MeHg exposure and concentrations in target tissues, with the assumption that higher MeHg exposure positively correlates with the risk of negative health outcomes. There is always a degree of imprecision with the use of hair-Hg as a biomarker for MeHg toxicity (Grandjean and Budtz-Jorgensen, 2007).

Despite the limitations of the research conducted, we provide evidence of the ability of dietary antioxidants to modify the neurotoxicity and toxicokinetics of MeHg from multiple testing scenarios. Dietary nutrient-MeHg interactions have significant implications for accurate risk assessment and warrants further exploration as a possible public health approach to risk reduction.
6.4 Concluding remarks

Although severe MeHg poisoning events, such as those of Minamata and Iraq, are not a current concern, low-level chronic developmental MeHg exposure remains a worldwide issue. Populations that rely on a diet contaminated with MeHg are particularly vulnerable as alternative sources of nutrients are often not available. Lack of public knowledge on healthy fish choices may also lead to excessive MeHg exposure. As fish and seafood are excellent sources of nutrients, it is important to have comprehensive public health approaches to mitigate the risk of MeHg exposure that are culturally appropriate and easy to understand. The determination of the risk associated with developmental MeHg exposure can often be obscured by confounding factors such as nutrient status. Understanding the influence and interactions of dietary nutrients on MeHg toxicity is a crucial aspect of accurate risk assessment strategies. This doctoral research contributes original information to the multidisciplinary field of ecotoxicology, with tools from molecular biology, epidemiology, ethnobotany, and physiology, in demonstrating the ability of dietary antioxidants to alter the outcomes of developmental MeHg exposure in a rat model, and the Hg body burden from fish consumption in a human population.
Appendix A. Food questionnaire and informed consent

¿Qué come usted?
Proyecto de Mercurio en Costa Rica

Instrucciones generales:
1. Lea y escuche la información sobre el proyecto.
2. Si decide participar, firme el consentimiento informado.
3. Responda a cada pregunta en el cuestionario de forma personal, lo mejor que pueda.
4. Las primeras 4 páginas serán removidas para proteger su confidencialidad.
5. Le enviaremos sus resultados personales y los resultados generales del proyecto por correo electrónico en agosto, 2009.
CONSENTIMIENTO INFORMADO

Un estudio sobre los niveles de exposición al mercurio en empleados y estudiantes de la Universidad Nacional

Introducción: El mercurio se encuentra en el medio-ambiente por las emisiones de volcanes y por medio de las actividades desarrolladas por el hombre, como la industria minera y la deforestación. En el medio-ambiente bacterias pueden transformar mercurio a metilmercurio. El metilmercurio es más alto en organismos que están arriba en la cadena alimentaria. O sea, los peces que comen otros peces. El pescado es un alimento muy saludable para el ser humano porque contiene muchos nutrientes saludables. Sin embargo, los seres humanos pueden ingerir metilmercurio cuando comen muy a menudo pescado contaminado. Los peces que pueden ser contaminados son peces que comen otros peces. En concentraciones altas, el metilmercurio es peligroso para el cerebro de fetos, niños y adultos. Es posible medir el nivel de mercurio en el pelo, y con esta información podemos compararlos con estándares internacionales e indicar si existe un riesgo de salud o no.

Objetivo: Evaluar los niveles de mercurio en el cabello de empleados y estudiantes de dos institutos de la Universidad Nacional de Costa Rica y compararlos con un cuestionario de alimentación.

Estimados: Se le preguntará llenar un cuestionario corto para obtener información sobre su dieta diaria. Además, se cortarán aproximadamente 20 pelos localizados en la zona de la nuca, área donde es poco visible o no es visible el corte. Las muestras de cabello se pondrán en una bolsa “ziplock” para su traslado seguro, con su correspondiente etiqueta y código.

Riesgos: La participación en este proyecto no presenta ningún riesgo para la salud.

Beneficios: Les informaremos personalmente sobre su nivel de mercurio en pelo y si este nivel es bajo, medio o alto en comparación con estándares formulados por la Organización Mundial de la Salud. Si usted tiene niveles medios o altos de mercurio le daremos recomendaciones nutricionales para disminuir los niveles de mercurio en su cuerpo.

Solicitud de colaboración y comunicación de resultados: Pedimos su colaboración en este proyecto. Usted es libre de decidir si quiere participar en el estudio o no. Le aseguramos que los resultados que obtengamos van a ser usados con mucho cuidado y confidencialmente. Excepto de usted y la coordinadora del estudio nadie va a saber su resultado personal.

Equipo de trabajo IRET-UNA: Paleah Black (estudiante de PhD), Aylín Castillo Ovares (Bach.) y Berna Natalia van Wendel (PhD) (responsable final).

Atentamente,

Paleah Black
Coordinadora del estudio

Aylín Castillo Ovares
Asistente del estudio

Berna van Wendel
Responsable final
CONSENTIMIENTO INFORMADO

Un estudio piloto sobre los niveles de exposición al mercurio en empleados y estudiantes de la Universidad Nacional

He leído la información sobre este estudio. He hablado con la investigadora y me ha contestado todas mis preguntas en un lenguaje comprensible para mí. Autorizo voluntariamente participar en el estudio. Tengo el derecho a negarme y a discontinuar mi participación, sin que esto me perjudique de ninguna manera.

Para cualquier pregunta puedo llamar a Palesh Black o a Berna van Wendel de la Universidad Nacional, al número o es posible escribir un email también, por cualquier consulta o por información al:

He recibido una copia de este consentimiento para mi uso personal.

____________________  ____________________  ____________________
Nombre y cédula  Firma  Fecha

____________________  ____________________  ____________________
Nombre y cédula del investigador  Firma  Fecha

____________________  ____________________  ____________________
Nombre y cédula del testigo  Firma  Fecha
Identificación:

Nombre: ____________________________

Fecha (dd/mm/aaaa): ____________________________

Sexo: □ Masculino □ Femenino

Edad: ____________________________

Fecha de nacimiento (dd/mm/aaaa): ____________________________

Curso/oferma: ____________________________

Número de teléfono o celular: ____________________________

Pueblo/ciudad donde vive: ____________________________

Correo electrónico: ____________________________

(para volver sus resultados)

Las primeras 4 páginas serán removidas para proteger su confidencialidad.
Por favor, indíque para todas las preguntas, si ha ingerido cierto producto durante los últimos 12 meses. En caso que sí, indique el NUMERO de veces por día, semana o mes.

1.- ¿Ha comido pescado (incluso todo tipo de pescado, atún en lata, ceviche, filetes, etc.)?
   □ No (pase a la pregunta 3) □ Sí: ___ por semana o ___ por mes o ___ por año

2.- ¿Con qué frecuencia ha consumido cada tipo de pescado?
   a) Corvina: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   b) Atún en lata: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   c) Atún no en lata: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   d) Tilapia: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   e) Trucha o salmón: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   f) Pargo: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   g) Marlin: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   h) Dorado: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   i) Pez espada: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   j) Chuleta de bohío: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   k) Ceviche: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año
   l) Otra. Escribe el tipo: □ Nunca  □ Sí: ___ por semana o ___ por mes o ___ por año

3.- ¿Había comido vegetales (incluye todo tipo de vegetales)?
   □ No (pase a la pregunta 5) □ Sí: ___ por día o ___ por semana o ___ por mes

4.- ¿Con qué frecuencia ha consumido cada tipo de vegetales?
   a) Vegetales de color verde oscuro (espinacas, brócoli, calabacín, etc.):
      □ Nunca  □ Sí: ___ por día o ___ por semana o ___ por mes
   b) Vegetales de color rojo o naranja (zanahorias, chile dulce, tomates, etc.):
      □ Nunca  □ Sí: ___ por día o ___ por semana o ___ por mes
Durante los últimos 12 meses...

5. ¿Ha comido frutas (incluyendo refrescos naturales)?
   □ No (pase a la pregunta 7)   □ Sí: ___ por día o ___ por semana o ___ por mes

6. ¿Con qué frecuencia ha consumido cada tipo de frutas?
   a) Banano: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   b) Mora o fresas: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   c) Piña: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   d) Limón o naranja: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   e) Mangos o mangas: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   f) Cas: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   g) Guayaba: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   h) Otra. Escribe el tipo: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes

7. ¿Ha consumido plantas medicinales o bebido té de hierba?
   □ No (pase a la pregunta 9)   □ Sí: ___ por día o ___ por semana o ___ por mes

8. ¿Con qué frecuencia ha consumido cada tipo de plantas medicinales o bebido té de hierba?
   a) Té de manzanilla: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   b) Té de tilo: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   c) Junípera: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   d) Hierba buena o menta: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   e) Núi: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   f) Ginko bioba: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   g) Añahaca o Romero: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
   h) Otra. Escribe el tipo: □ Nunca   □ Sí: ___ por día o ___ por semana o ___ por mes
Durante los últimos 12 meses...

9.- ¿Cuántas tazas de café, té negro, o té verde ha bebido (incluye las botellitas de té frío)?
   a) Café: □ Nunca □ Sí: ___ por día o ___ por semana o ___ por mes
   b) Té negro (caliente o frío): □ Nunca □ Sí: ___ por día o ___ por semana o ___ por mes
   c) Té verde (caliente o frío): □ Nunca □ Sí: ___ por día o ___ por semana o ___ por mes

10.- ¿Ha comido alimentos integrales (incluye avena, pan integral, pasta de trigo entero, arroz integral, etc.)?
     □ No □ Sí: ___ por día o ___ por semana o ___ por mes

11.- ¿Ha comido nueces (marrasquino, avellanas, marrón, nueces de Brasil, almendras, etc.)?
     □ No □ Sí: ___ por día o ___ por semana o ___ por mes

12.- ¿Ha comido huevos?
     □ No □ Sí: ___ por día o ___ por semana o ___ por mes

13.- ¿Ha bebido alcohol (cerveza, vino, guaro, licor)?
     □ No □ Sí: ___ por día o ___ por semana o ___ por mes

14.- ¿Ha fumado cigarrillos de tabaco?
     □ No □ Sí: ___ por día o ___ por semana o ___ por mes

15.- ¿Ha tomado vitaminas o suplementos (multivitaminas, vitaminas pautales, vitamina C, hierro, selenio, zinc)?
     □ No □ Sí: ___ por día o ___ por semana o ___ por mes
     ¿Qué tipo de vitaminas o suplementos?: _______________________

16.- ¿Ha usado un permanente (rizado) para su cabello durante los últimos 12 meses?
     □ No □ Sí: ¿Cuánto? ______

17.- ¿Usted tiene calzas grises? (Si no sabe, use un espejo para que usted se cuente o puede ir al baño)
     □ No □ Sí: ¿Cuántas? ______

18.- ¿Ha estado resfriado, teniendo gripe u otras infecciones durante los últimos 12 meses?
     □ Ninguna vez □ Sí: ___ por año

¡Muchas gracias por su participación!
Appendix B. Other contributions to research and development

1) Conference proceedings (a Oral presentation, b Poster presentation)


2) Other published peer-reviewed articles


Aylin Castillo, Juan Valdes, Jose Sibaja, Ilena Vega, Rosa Alfaro, José Morales, Germain Esquivel, Elisa Barrantes, Paleah Black and David Lean. (In press). Seasonal and Diel Patterns of Total Gaseous Mercury Concentration In Atmospheric Air of the Central Valley of Costa Rica. J. Applied Geochemistry.

3) Extracurricular training

- Developmental toxicology and risk assessment, Health Canada (2 day course, S2009)
- Survey design, use, and data analysis, Health Canada (3 day course, W2009)
- Methods for assessing CNS toxicity, SETAC & University of Ottawa (2 day workshop, F2008)
- Spanish language training, Health Canada (2hr/wk, S2007 to W2008)
- Statistical analysis for biological data, Health Canada (3 day course, W2007)
- Molecular Mechanisms of Apoptosis (BCH8109), University of Ottawa (Audited, F2007)
- GeneSpring statistical analysis of genomic data, Health Canada (3 day course, W2006)
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