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Fate and developmental effects of dietary uptake of methylmercury in *Xenopus tropicalis* tadpoles

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In partial fulfillment of the requirements for the
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Abstract

To investigate the effects of methylmercury (MeHg) on development, *Xenopus tropicalis* tadpoles were exposed to MeHg diets with concentrations of 0.42 (control), 44 (low), and 270 (high) ng/g wet weight. Increased mortality, decreased metamorphosis, increased size, and a greater number of days between tadpoles reaching metamorphosis were observed in the high MeHg group, suggesting disruption to the thyroid axis. Triiodothyronine levels, however, were not significantly different between groups. In both the control and low MeHg groups, total Hg and MeHg body burdens increased rapidly, reached a plateau and eventually declined with a lower percent MeHg body burden. In the high MeHg group, body burden concentrations increased throughout the experiment. This study demonstrates that at low dosages of MeHg, elimination may prevent toxic exposure whereas at high dosages, demethylation and selective excretion mechanisms may be overwhelmed and disruption of development or death may occur in tadpoles.

Résumé

Des têtards *Xenopus tropicalis* ont étés exposés à une diète contenant du méthylmercure (MeHg) à des concentrations de 0.42 (contrôle), 44 (niveau bas) et 270 (niveau élevé) ng/g (poids humide), afin d'examiner les effets du MeHg sur leur développement. Dans le groupe exposé au niveau élevé de MeHg, nous avons observé une augmentation de mortalité, une diminution du nombre d'individus ayant atteint la métamorphose, une augmentation de taille et un plus grand nombre de journées entre les têtards ayant atteint la métamorphose, suggérant une perturbation du système thyroïdien. Par contre, les niveaux de triiodothyronine n'étaient pas significativement différents entre les traitements. Dans les groupes contrôle et niveau bas de MeHg, les niveaux de Hg total et de MeHg accumulés dans le corps ont augmenté rapidement, ayant atteint un plateau qui a baissé par la suite avec un pourcentage plus bas de MeHg accumulé dans le corps. Chez le groupe exposé au niveau élevé de MeHg, les concentrations de MeHg accumulées dans le corps ont augmenté au cours de l'expérience. Cette étude démontre qu'à des doses faibles de MeHg, les mécanismes d'élimination peuvent prévenir l'exposition toxique alors qu'à des doses elevées, les mécanismes d'excrétion sélective et de déméthylation peuvent être bouleversés, provoquant des perturbations du développement ou la mort des têtards.

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List of Abbreviations

ACTH - Adrenocorticotropin
Al_2O_3 - Aluminum oxide
ANOVA - Analysis of variance
BPA - Bisphenol A
BrCl - Bromine chloride
$Ca(OH)_2$ - Calcium hydroxide
CH_3Cl - Chloroform
CRF - Corticotropin releasing factor
CS - Corticosteroids
$CuSO_4$ - Copper (II) sulphate
CVAFS - Cold vapour atomic fluorescence spectroscopy
DCM - Dichloromethane
D1 - Type I iodothyronine deiodinase
D2 - Type II iodothyronine deiodinase
D3 - Type III iodothyronine deiodinase
DOC - Dissolved organic carbon
DOM - Dissolved organic matter
DORM-2 - Dogfish muscle standard reference material
EIA - Enzyme immunoassay
ER - Estrogen receptor
EtOH - Ethanol
G - Gosner developmental stage
GC AFS - Gas chromatograph atomic absorption fluorescence spectrometry
GH - Growth hormone
GLM - General linear model
HCG - Human chorionic gonadotropin
HCL - Hydrochloric acid
HDPE - High density polyethylene
Hg - Mercury
KBr - Potassium bromide
KOH - Potassium hydroxide
LC50 - Lethal Concentration 50%
MDL - Method of detection limit
MeHg - Methylmercury
MeHgCl - Methylmercury chloride
MeOH - Methanol
MQ - Milli-Q® polished water
mRNA - Messenger ribonucleic acid
MS-222 - Ethyl 3-aminobenzoate methanesulfonate salt
MT - Metallothionein
Na_2CO_3 - Sodium carbonate
Na_2SO_4 - Sodium sulphate
$Na_2S_2O_3$ - Sodium thiosulphate
NF - Nieuwkoop and Faber developmental stage
NH_4OH - Ammonium hydroxide

PBS - Phosphate-buffered saline
PDE - Pentabromodiphenyl ether
PRL - Prolactin
PTU - Propylthiouracil
SH - Sulfhydryl group
T_3 - Triiodothyronine
T_4 - Thyroxine
TH - Thyroid hormone
THg - Total mercury
TR - Thyroid hormone receptor
TRE - Thyroid response element
TRH - Thyrotropin releasing hormone
TSH - Thyrotropin
UV - Ultraviolet

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CHAPTER 1

General introduction

1.1 Rationale

Mercury (Hg) is released into the environment by both natural and anthropogenic sources. When Hg enters wetlands, oceans, lakes, and rivers it is converted into methylmercury (MeHg), the most toxic and biologically active form of Hg, by aquatic biota and also through abiotic chemical processes (Newman and Unger 2003, Celo et al. 2005). MeHg is a chemical of concern because it is ubiquitous, transient, and has the ability to accumulate in the tissues of many organisms. These characteristics allow it to biomagnify throughout aquatic food chains. In humans, exposure to Hg due to the consumption of fish is known to cause neurotoxicity (Davidson et al. 2004). Therefore, human health concerns have traditionally driven research into MeHg toxicity and global Hg cycling.

MeHg is found naturally in amphibian habitats as Hg methylation occurs in sediments and flooded soils as well as within reservoir systems and wetlands (Newman and Unger 2003). Some amphibians may spend their entire life cycle in aquatic environments, therefore exposure to high levels of MeHg due to inputs from anthropogenic sources such as the combustion of fossil fuels, chlor-alkali plants, pulp mills, and mining activities may increase mortality during development (Eisler 2006). Exposure to low levels may have physiological consequences that contribute to morbidity and mortality within a population (Gibson 2005). This requires careful examination because the worldwide decline and disappearance of amphibian populations has been documented since the 1960’s (Houlahan et al. 2000). Although it is unlikely to be the

primary cause of these declines, MeHg toxicity has been proposed as a possible contributor (Bank et al. 2005, Bank et al. 2007).

It is well known that amphibians are important indicators of ecological health as they are sensitive to many changes in the environment. They are also useful models for toxicological studies as some stages of amphibian development mirror stages of development in many other organisms, including humans (McEwen et al. 1991, Tata 1993). In all developing vertebrates, post-embryonic development, neurogenesis, and maturation of the central nervous system are highly dependant on the actions of thyroid hormones (THs) derived from the hypothalmo-pituitary-thyroid axis (Tata 2006). Any interference with this endocrine axis can have negative effects on the developing organism. Although the exact biochemical mechanism by which MeHg induces toxicity is not fully understood (Clarkson 1998, Kang et al. 2006), it is known that in humans, the fetus appears to be more sensitive to those effects than the adult (Kudo et al. 1998). This increased developmental sensitivity is also apparent in many other organisms, including anuran tadpoles (Unrine et al. 2004). By examining the effects of MeHg in model organisms such anurans, it may be possible to elucidate the mechanisms responsible for its toxicity across species boundaries and determine if the potential for detoxification mechanisms during development exist.

Investigation is required to determine how MeHg is accumulated by amphibians. Unfortunately, most studies have involved sampling amphibians in the wild. Therefore, they have had limited success in predicting bioaccumulation as Hg concentrations in tissues could not be related to specific exposure concentrations. Furthermore, many of these studies have focused on adult anurans, which are often partially terrestrial whereas

most developing larvae are aquatic and are therefore likely to be exposed to higher Hg concentrations. A recent study, however, exposed *Rana sphenocephala* (Southern leopard frog) larvae to experimental diets containing 100, 500, and 1,000 ng/g dry wt. of total mercury (THg) for the entire larval period to determine the relationship between inorganic and organic Hg in tadpole diets (Unrine and Jagoe 2004). Analysis of ground tadpole tissue revealed that MeHg concentration increased with THg concentration, but the proportion of MeHg to inorganic Hg decreased as THg concentration increased. The impacts of this relationship of accumulation remain to be explored as whole body levels of Hg were not measured and results were not adjusted for body mass dilution resulting from tadpole growth or potential differences in weight between groups. Hg elimination and detoxification were also not addressed.

Few studies have investigated the potential of organic Hg to act as an amphibian endocrine disruptor. Evidence that MeHg has the capacity to interfere with deiodinase activity (Watanabe et al. 1999, Mori et al. 2006a, Mori et al. 2006b, Mori et al. 2007), which is essential for normal functioning within the TH axis, indicates that MeHg may be able to disrupt the normal progression of development. Imbalances in TH levels resulting from changes to components of the TH axis (e.g., deiodinases) have the capacity to disrupt growth, tissue differentiation, and metamorphic rate in tadpoles (Denver et al. 2002), all of which could impact the survival of the affected organisms. Therefore, more investigation is required to determine the effects of MeHg on the TH axis during development.

1.2 Hypotheses and objectives

My research focused on the effects of a chronic, sublethal dietary MeHg exposure on the development of the laboratory model species *Xenopus tropicalis* (Western clawed frog). I hypothesized that tadpoles would accumulate measurable levels of THg and MeHg throughout the larval period. I predicted that initial MeHg uptake by tadpoles would be high, and would be followed by a period of MeHg assimilation and an eventual steady state between MeHg uptake and decontamination and/or excretion. Most organisms have a strong capacity for MeHg bioaccumulation and a low capacity for MeHg excretion (when compared to inorganic Hg). Therefore, differences between THg and MeHg uptake were predicted to be low in all treatment groups. I also predicted that MeHg would disrupt amphibian development and metamorphosis, and that the extent of these disruptions would be related to exposure levels. Low level MeHg ingestion would increase rates of metamorphosis resulting in tadpoles that are smaller at metamorphic climax. Ingestion of a higher level of MeHg would inhibit development, resulting in high mortality and low rates of metamorphosis. Additionally, changes in growth, behaviour, and body morphology were predicted to be dependent on the treatment level of MeHg ingestion. Finally, I predicted that exposure to MeHg would disrupt the TH axis and that differences in TH levels would be observed between treatment groups, with the greatest changes appearing in the high treatment group.

My goals for this research are: 1) to characterize patterns of MeHg accumulation and excretion in amphibian tadpoles undergoing development; and 2) to examine the effects of MeHg exposure on rates of development and metamorphosis and link these effects to the TH axis.

1.3 Mercury cycle and methylmercury

Inputs of Hg into the environment arise from both natural and anthropogenic sources, with the latter growing in importance since the early 20th century (Newman and Unger 2003). Natural sources include emissions from volcanoes, degassing of the earth's crust, and volatization from oceans (Boening 2000, Baeyens et al. 2003). Anthropogenic sources include, but are not limited to, mining operations, copper, lead, and zinc smelting and through the burning of fossil fuels (Boening 2000, USEPA 2004). Globally, a significant fraction of Hg inputs originate from anthropogenic sources and it has been estimated that overall deposition has increased by a factor of at least three since the Industrial Revolution (Biester et al. 2007, Lindberg et al. 2007). There is a scientific consensus that approximately two-thirds of the global pool of Hg is derived from human activities, whereas approximately one-third originates from natural sources (as stated in the Madison Declaration on Mercury Pollution 2007).

In the atmosphere, Hg is transported as the volatile organic species Hg^0 (Figure 1.1). When Hg^0 is converted to Hg^{2+} through oxidation, it can be rapidly taken up in rain water, snow, or absorbed onto small particles and be subsequently deposited in the environment through "wet" or "dry" deposition (Morel et al. 1998). Wet and dry deposition of Hg is generally in the oxidized form of Hg^{2+} and, to a small degree, MeHg (Morel et al. 1998). In terms of inputs of Hg into aquatic environments, terrestrial soils are probably the most significant contributor of Hg to surface water, with a significant amount of MeHg originating from the terrestrial watershed (Gabriel and Williamson 2004). It has been estimated that up to 60% of the atmospherically deposited Hg that reaches lakes originates from the associated terrestrial watershed (Grigal 2002). Once

oxidized Hg^{2+} enters a watershed, many factors can affect speciation such as pH, ionic strength, redox potential, and the concentrations of dissolved organic matter (DOM), dissolved oxygen, sulfide, and suspended solids in solution (Gabriel and Williamson 2004). Ionic species bound to DOM such as MeHg are reduced through bacterial processes or through photodegradation by ultraviolet (UV) radiation resulting in Hg being converted back to Hg^{2+} and Hg^0 forms (Morel et al. 1998).

Deposition of Hg can become problematic when fallout from the atmosphere occurs on aquatic ecosystems, where the conversion of a small portion of the inorganic Hg^{2+} to MeHg at the sediment water interface can result in high levels of MeHg in tissues of biota at the top of aquatic food webs (Swain et al. 1992, St. Louis et al. 1996, Mason et al. 2000, Hall et al. 2005, Hall et al. 2008). In aquatic environments, two general methylation pathways exist: chemical methylation (abiotic processes) and microbial metabolism (biotic processes). While it is widely accepted that biotic processes account for most or all environmental MeHg, a number of investigators have also provided evidence for abiotic Hg methylation (Cerrati et al. 1992, Rosenkranz et al. 1997, Celo et al. 2005). Chemical reactions involving small organic molecules such as methyliodide and dimethylsulfide, and larger organic components of dissolved organic matter such as fulvic and humic acids, as well as transmethylation reactions involving organometallic complexes such as methylcobalamin, methyllead, or methyltin compounds have also been implicated in Hg methylation and demethylation (Celo et al. 2005). Biotic Hg methylation and demethylation by bacteria such as methanogens has been extensively studied. Although microbial methylation has traditionally been associated with the actions of sulfate-reducing bacteria (Newman and Unger 2003), recent studies have

demonstrated that iron-reducing bacteria also have the capacity to methylate Hg and produce amounts equal to those produced by sulphate-reducing bacteria (Fleming et al. 2006). Biotic methylation takes place under anaerobic conditions and is subject to the effects of many chemical and physical conditions such as water pH, salinity, and amount of dissolved organic carbon (DOC) (Amirbahman et al. 2002; Table 1.1).

Wetland sediments, in particular, possess many environmental characteristics that promote methylation and are recognized as hot spots for MeHg production (Hall et al. 2008). They contain large colonies of sulphate-reducing bacteria and water concentrations of MeHg are positively correlated with the number of wetlands within a lake's catchment area (St. Louis et al. 1996). Wetlands appear to convert a small but significant fraction of deposited Hg into MeHg which can be exported to nearby water bodies. This is due to characteristics that wetlands possess such as a high amount of organic matter (St. Louis et al. 1994), anoxic conditions, sulfate-reducing bacteria within sediments (Langer et al. 2001), and DOM to protect MeHg from UV photo-oxidation (Crump 2001). Wetlands are ideal habitats for amphibians and MeHg produced in wetlands has the capacity to bind to DOM, a food sources for some developing tadpoles. Increased levels of MeHg in these habitats can lead to increased exposure during development and toxic effects.

In nature, Hg concentrations in tadpole diet items can vary depending on the item and the location of sampling. For example, Plourde et al. (1997) found that in natural Québec lakes, suspended particulate matter contained between 10 and 20 ng MeHg/g dry wt. and zooplankton contained between 60 to 170 ng MeHg/g dry wt. MeHg. Unrine and Jagoe (2004) have found MeHg levels ranging from 8 to 57 ng/g dry wt. in periphyton

collected from several environmental sites, including a constructed wetland in South Carolina. Westcott and Kalff (1996) measured an average of 95 ng/g dry wt. MeHg in zooplankton in clear water lakes and an average of 289 ng/g dry wt. MeHg in zooplankton from brown water lakes in south central Ontario. Sarica et al. (2005) found that small-mouth bass (*Micropterus dolomieu*) carcasses from lakes free of point sources of Hg contained 690 ng/g dry wt. MeHg in small fish, and up to 1370 ng/g dry wt. MeHg in large fish. In a meta-analysis of Hg concentrations in freshwater fish across North America, Kamman et al. (2005) found that fish sampled from unpolluted lakes ranged from 196 to 746 ng/g wet wt. of THg.

Aquatic environments that are contaminated, such as those receiving Hg-contaminated effluent from industry, have been found to contain tadpole diet items with extremely elevated levels of MeHg. For example, Wilken and Hintelmann (1991) found that MeHg concentrations in suspended particulate matter in the Elbe river in Northern Germany, one of the most Hg polluted rivers in the world due to inputs from a chlor-alkali plant, were as high as 2700 ng/g dry wt. In periphyton from industrially contaminated streams in Tennessee, THg concentrations as high as 50000 ng/g dry wt. have been measured and grazing fish have been found to have MeHg levels of nearly 250 ng/g wet wt. (Hill et al. 1996). In the marine environment of Minamata Bay, Kyushu Japan, Hg levels in phytoplankton have been measured to be as high as 320 ng/g dry wt., zooplankton as high as 1100 ng/g dry wt. (Nishimura and Kumagai 1983), filter feeding mollusks as high as 60000 ng/g dry wt. (Fujuki 1980), and fish (*Choerodon azurio*) as high as 309000 ng/g dry wt. (Fujuki 1963) all due to contamination by a chlor-alkali plant. In Canada, fish sampled from the English-Wabigoon River System in

Northwestern Ontario, which was also contaminated by wastes from a chlor-alkali plant, contained up to 27800 ng/g wet wt. of THg (Eisler 2006). Any of the aforementioned (freshwater) organisms are potential diet items for tadpoles (in the case of fish exposure would result from feeding on carcasses). Therefore, the consumption of those items containing high levels of MeHg could lead to MeHg bioaccumulation, and in turn have detrimental effects on growth, behaviour, and development as discussed below (see *1.5 Methylmercury and organisms*).

1.4 Amphibian metamorphosis

Amphibian metamorphosis is very complex because it is regulated by external environmental factors and internal hormonal processes. Metamorphic rates and larval development are influenced by, and will vary in relation to, changes in environmental habitat (Hayes 1997). Biotic factors that influence amphibian development include, but are not limited to: food availability, predation, population density, and inter- and intraspecific chemical signaling (Taylor and Scott 1997, Denver et al. 2002). Abiotic factors include water temperature, photoperiod, and pond duration (Denver et al. 1998a, Denver et al. 2002, Tata 2006). Increasing temperature, decreasing food, overcrowding, pond evaporation, and increased predation have all been demonstarted to accelerate metamorphosis (Hayes 1997). Environmental contaminants can also disrupt development by delaying or accelarating metamorphic rate, resulting in metamorphs that may be incapable of survival at their resulting size (Bridges 2000).

The two pathways that are thought to be involved in the process of tadpole metamorphosis include the corticotropin releasing hormone (CRH) pathway and the

thyrotropin releasing hormone (TRH; also called thyrotropin releasing factor - TRF) pathway (Denver 1998b, Tata 1999, Shi 2000, Denver et al. 2002; Figure 1.2). The CRH pathway controls both the thyroid and interrenal axes by directly stimulating pituitary thyrotropin (TSH) and adrenocorticotropin (ACTH) (Denver 1998b). The THs thyroxine (T_4) and triiodothyronine (T_3) are the primary morphogens controlling metamorphosis and their production is controlled by TSH. All changes occurring during larval development are induced by T_4; however, it is enzymatically converted to T_3 in the thyroid gland and within target tissues. T_3 has ten times or greater the biological potency of T_4 (Denver et al. 2002). Plasma T_3 levels rise during metamorphosis and reach a peak at climax. In addition to T_3, plasma levels of corticosteroids (produced by the interrenal glands in response to ACTH) rise during metamorphosis and have been shown to synergize with T_3 to promote metamorphosis (Denver 1998a).

For metamorphosis to proceed, feedback systems must be in place to maintain control over hormone levels. The conversion of THs to active and inactive forms by deiodinase enzymes plays an important role in regulating TH levels in various tissues at different stages of tadpole development (Brown 2005). Amphibians possess three types of deiodinase enzymes. Type I iodothyronine deiodinase (D1) was recently found to exist in amphibians and although it is thought that D1 is capable of both activating and inactivating TH, its role in development and metamorphosis remains to be fully characterized (Kuiper et al. 2006; Dubois et al. 2006). Type II iodothyronine deiodinase (D2) converts T_4 to T_3, making it biologically active and capable of binding with thyroid receptors (Huang et al. 2001). Type III iodothyronine deiodinase (D3) on the other hand, inactivates T_3 by removing an iodine molecule from its inner ring producing T_2, and

inactivates T_4 by converting it to the biologically inactive reverse T_3 (Huang et al. 2001). During the activation of metamorphosis, there is a measurable increase in D2 that enhances formation of T_3 from T_4, as well as a decrease in D3 activity (Norris 1997). Regulation through this pathway ensures that the amount of biologically active TH is maintained at appropriate levels during each stage of metamorphosis.

Thyroid hormone receptors (TRs) are members of an evolutionarily highly conserved supergene family of steroid/TH/retinoid nuclear receptors that function as ligand-inducible transcription factors (Tata 1999). In all vertebrates TRs are encoded by at least two genes (α and ß). *Xenopus laevis* (African clawed frog), for example, possesses two TRα and two TRß genes (owing to its pseudotetraploidy), each of which are expressed during metamorphosis (Denver 1998b, Brown and Cai 2007). Unliganded TRs are located in the nucleus where they are bound by chromatin which may suppress gene expression. Unlike other nuclear receptors, an unliganded TR acts as a strong repressor with the repression being relieved by the ligand (Tata 2000). When TRs bind T_3, a dimerization of TRs will begin transcription at the thyroid response elements (TREs) of the controlled gene.

During metamorphosis, TR autoinduction has been shown to be triggered by the direct interaction between the receptor protein and the TREs in the promoter of its own gene (Tata 2000). TRß is directly responsive to TH. Therefore, as levels of T_3 increase, a rapid and substantial upregulation of the TRß messenger ribonucleic acid (mRNA) (up to 50-fold) occurs in all premetamorphic tissues (Ulisse et al. 1996, Shreiber et al. 2001). It is thought that T_3 may regulate the expression of its own receptor genes and that this may

facilitate the activation of secondary gene transcription leading to tissue specific development based on metamorphic requirements (Tata 1999).

Amphibian development and metamorphosis are under the intricate control of the hypothalamo-pituitary-thyroid axis and disruption to any component of this axis could lead to negative effects on the fitness of the organism. Although MeHg is known to have effects on other organisms, both at acute and sublethal levels of exposure, its effects on amphibians have not been fully considered. More investigation is required to evaluate the effects of MeHg on amphibian development and metamorphosis.

1.5 Methylmercury and organisms

MeHg toxicity has been demonstrated to affect many organisms by presenting both lethal and sublethal effects. The aim of this section is to review the mode of action of Hg toxicity, including known mechanisms of biochemistry (accumulation and excretion) as well as physiological, behavioral, and cellular level effects for various organisms across different taxa.

1.5.1 Mercury biochemistry

The primary chemical targets of Hg conjugation in biological systems are the thiol groups that are located within the active catalytic sites of many enzymes. These groups play fundamental structural and functional roles in protein chemistry and Hg compounds react specifically with the active sulfhydryl (SH) groups that they contain (Aschner and Syversen 2005). As SH groups are ubiquitous within cells, the binding of Hg has the capacity to alter many different functions and binding is strongly associated with protein

inactivation and consequent cell death in the central nervous system (Crespo-Lopez et al. 2007, de Melo Reis et al. 2007). The affinity of MeHg for the anionic form of SH groups is also extremely high and whenever a MeHg compound has been identified in biological media it has been complexed to SH-containing ligands (Carty and Malone 1979). Complexes of MeHg with the amino acid cysteine and the tripeptide glutathione have been found in the blood of rabbits and humans, and glutathione complexes have been found in the brain, liver, and bile of rats (Aschner and Syversen 2005).

MeHg has the capacity to cross the blood-brain and placental barriers when compared to inorganic Hg compounds. In fact, organomercurials are more completely absorbed, are more soluble in organic solvents and lipids, pass more readily through biological membranes, and are not excreted as rapidly as inorganic forms of Hg (Eisler 2006). When dietary MeHg is consumed, up to 90% is absorbed by the gastrointestinal tract as it becomes bound to proteins that allow it to cross the intestinal membrane (Clarkson 1993, Myers and Davidson 1998). When MeHg attaches to the thiol ligand of cysteine, a complex is formed with a structure that mimics the large neutral amino acid methionine, which is able to enter cells and cross membrane barriers using neutral amino acid carriers (Clarkson et al. 2007). Once the intestinal membrane has been crossed, bound MeHg enters the bloodstream and is distributed to all tissues in the body of the exposed organism. In the adult brain, MeHg accumulates in astrocytes and interferes with glutamate uptake resulting in high extracellular glutamate (Aschner and Syversen 2005). Glutamate is an abundant neurotransmitter and can be neurotoxic since high levels have been implicated in the pathogenesis of stroke, amyotrophic lateral sclerosis, epilepsy, and other neurological diseases (Anderson and Swanson 2000).

In pregnant women, MeHg readily crosses the placental barrier and has a high affinity for fetal hemoglobin (Myers and Davidson 1998). Research into MeHg transfer across the placenta of pregnant rats has shown that MeHg is actively transported as a cysteine conjugate that also mimics methionine, via the neutral amino acid carrier system (Kajiwara et al. 1996). Once in the developing brain, MeHg has the capacity to disrupt normal pathways of development such as the way in which neurons move from the site of germination to the final site of function, the formation of interneuronal contacts, and the apoptotic death of neurons (Anderson and Swanson 2000). As all of these events are necessary for normal development, MeHg may irreparably harm the central nervous system, making the developing brain far more vulnerable than the adult brain.

1.5.2 Physiological and behavioural effects of methylmercury

A great deal of information exists concerning the human toxicology of MeHg and more studies continue to investigate its physiological effects. Exposure to MeHg, however, can also have physiological effects on many organisms other than humans. Research conducted on non-human mammals, birds, and fish has demonstrated various symptoms and effects due to MeHg poisoning as described below.

Piscivorous wildlife species are routinely exposed to Hg in their natural environments at levels that may cause adverse behavioural and physiological outcomes. MeHg toxicity is of particular concern for these organisms because it is the most relevant form of Hg to which they are exposed; more than 95% of the Hg in fish is in the organic form (Basu et al. 2007). Mink and otter are the mammalian wildlife species for which the greatest amount of information exists regarding Hg exposure and toxicity, and data from

several studies indicate that the consumption of 1 μg/g wet wt. of MeHg leads to neurotoxicity and death in adult mink and otter (Scheuhammer at al. 2007). Initial studies investigating the acute effects of dietary MeHg exposure on *Mustela vison* (American mink) demonstrated that individuals exposed to 5 μg/g wet wt. of MeHg per day exhibited decreased coordination, loss of balance, and anorexia or ataxia within 25 days. Eventually, symptoms worsened and once ataxia, paralysis, tremors, and convulsions were observed, death usually ensued within 48 hours (Aulerich et al. 1974). In a study by Wren et al. (1987), mink fed 1 μg/g wet wt. of MeHg exhibited lethargy, weakness in the hind limbs, and anorexia after 3 months of exposure. These symptoms eventually progressed to tremors and convulsions at which point the animals either died or were euthanized. Brain histology indicated the presence of brain lesions, abnormal blood vessels and neural degeneration of the parietal and frontal lobes.

Behavioural, reproductive, and anatomical endpoints have also been investigated for birds exposed to MeHg and the toxicology is similar to that found in mammals (Wolfe et al. 1998). In adults, MeHg concentrations of 15 μg/g wet wt. found in tissues have been associated with MeHg intoxication and death (Scheuhammer at al. 2007). In *Anas platyrhynchos* (mallard duck), paralysis of the legs was demonstrated after 7 weeks of exposure to 10 μg/g wet wt. of MeHg and hatching success was found to be significantly lower for females (Heinz and Hoffman 1998). In *Ardea albus* (great egret) a high dose of 5 μg/g wet wt. of MeHg induced sub-acute toxicosis after 10–12 weeks in nestlings exposed to MeHg for 14 weeks (Spalding et al. 2000a). Appetite and weight index (weight/bill length) declined after only 9 weeks and these changes were also apparent in individuals exposed to a low dosage of 0.5 μg/g. In a further study involving

A. albus nestlings exposed to 0.1 and 5 μg/g wet wt. of MeHg, low dosed birds had lower packed cell volumes, dingy feathers, increased lymphocytic cuffing in a skin test, increased bone marrow cellularity, decreased bursal wall thickness, decreased thymic lobule size, fewer lymphoid aggregates in lung, increased perivascular edema in lung, and decreased phagocytized carbon in lung (Spalding et al. 2000b). High dosed birds became severely ataxic and presented hematologic, neurologic, and histologic changes with severe lesions appearing in immune and nervous system tissues.

In fish, exposure to MeHg has been shown to have various physiological and behavioural effects. For example, environmentally relevant concentrations of dietary MeHg have been shown to impair reproduction in *Pimephales promelas* (fathead minnow). In a study by Hammershmidt et al. (2002), dietary MeHg was shown to delay spawning. The total number of days to spawning was positively correlated with the concentration of total Hg in the carcasses of fish. MeHg also decreased the reproductive rate of adults when they were exposed to dietary concentrations commonly encountered by predatory fishes in aquatic systems. When *Thymallus thymallus* (grayling) embryos exposed to different concentrations of MeHg (0.16, 0.8, 4.0 and 20 mg/L added to aquarium water) during the first ten days of development were evaluated for sublethal effects on foraging behaviour three years later, exposed groups were found to have impaired feeding efficiencies and reduced competitive abilities when compared to the control group (Fjeld et al. 1998). Juvenile *Orthodon microlepidotus* (blackfish) fed diets containing either 22.2 or 55.5 μg/g dry wt. of MeHg did not show differences in food consumption when compared to controls. By 35 days of exposure, however, the high treatment group had a significantly depressed growth rate when compared to the control

group and by 70 days both the medium and high groups had significantly lower growth rates (Houck and Cech 2004).

Few studies have examined the effects of MeHg exposure on amphibians and of those that have been conducted, most have involved levels much higher than would likely be encountered in the natural environment. For example, Chang et al. (1974) investigated the effects of various concentrations of MeHg chloride (MeHgCl) added to water on *Rana pipiens* (Northern leopard frog) tadpoles during late development. Tadpoles chronically exposed to concentrations of 0.001 to 0.01 mg/L demonstrated lethargy within 24 hours and metamorphosis did not occur even after three to four months had passed. Individuals exposed to 0.05 to 0.1 mg/L exhibited abnormal swimming behaviour and difficulty breathing within 24 hours. All tadpoles in this group succumbed to treatment after 48 hours and at the time of death all individuals exhibited distention and swelling of their bodies. Those in the 0.5 to 1 mg/L treatments also displayed the aforementioned symptoms and died after 24 hours. A further experiment investigated the effects of MeHg injections on tadpoles kept in clean water. Individuals were injected with 0.025 mg of MeHgCl five times a day for ten days. The researchers observed extensive swelling and distension of the hind legs after one week of MeHg administration. Further examination demonstrated severe edema of the legs with separation of the muscle fiber bundles. The liver was heavily pigmented and many renal tubules were replaced by hyperchromic cells. As the levels of MeHg that tadpoles normally encounter in water in nature are in the ng/L range, and this study exposed tadpoles to extremely high levels of MeHg in the mg/L range, the results of this study should be interpreted with caution as they are not representative of what would normally be observed in amphibian habitats.

A few recent studies have exposed tadpoles to environmentally relevant levels of MeHg. Unrine et al. (2004) have investigated the effects of oral exposure to lower levels of Hg on *R. sphenocephala* larvae. As it has been shown that in natural environments tadpoles are exposed to Hg and MeHg through diet and that accumulation through other sources is negligible (Unrine and Jagoe 2004), tadpoles in this experiment were orally exposed to Hg. Larvae were fed food spiked with one of three concentrations of Hg, 100, 500 and 1000 ng/g wet wt. (low, medium and high), beginning at Gosner (G) stage 25 (beginning of feeding; Gosner 1960) until metamorphosis. The most rapid development occurred within the medium and high treatment groups, corresponding to approximately 27 and 49 ng/g dry wt. MeHg dietary exposure, respectively. Days post-hatch to full hindlimb and forelimb development were significantly shorter in the high treatment group than in controls, and mean tadpole mass was significantly higher in the high treatment group when compared to controls at 55 days post hatch.

Gibson (2005) also investigated the effects of sub-chronic MeHg exposure on three species of amphibians, *Bufo americanus* (American toad), *R. pipiens* and *X. tropicalis*, to determine the lethal concentration of MeHg to kill 50% of the tadpoles (LC50) and species sensitivity differences. This study laid the foundations for my thesis research. Tadpoles of each species were orally exposed to MeHgCl ranging in concentration from 1 ng/g to 1000 ng/g wet wt. shortly after the beginning of feeding (G stage 25 for both *B. americanus* and *R. pipiens*, G stages 27-30 for another group of *B. americanus* tadpoles; Nieuwkoop and Faber (NF; 1994) stage 47 for *X. tropicalis*). On day 33 of exposure *B. americanus* tadpoles from the G25 treatment group had an LC50 of 323 ng/g and *B. americanus* tadpoles from the G27-30 group had an LC50 of 707 ng/g.

The results of the second group were found to be significantly different than the first showing greater sensitivity in this species to MeHg exposure at earlier stages of development. On the same day of exposure (day 33), G25 *R. pipiens* tadpoles had an LC50 of 371 ng/g and NF47 *X. tropicalis* tadpoles had an LC50 of 926 ng/g. LC50 results for *X. tropicalis* tadpoles were significantly different from those of both groups of *B. americanus* and *R. pipiens*.

In addition to the LC50 study, Gibson (2005) also conducted a chronic, low level MeHg ingestion study using *X. tropicalis* tadpoles. Tadpoles were exposed to one of three treatments, a control treatment containing no MeHgCl, 50 ng/g MeHgCl or 500 ng/g MeHgCl. Exposure was initiated for tadpoles ranging in stage from NF 46 to NF 54^+ and was ended once forelimb emergence occurred. Time to metamorphosis was found to be 140% higher in the 50 ng/g treatment than in the control group. Metamorphosis in the 500 ng/g treatment was 84% of that in the controls. The 500 ng/g treatment had low metamorphosis throughout the experiment, never exceeding either the 50 ng/g treatment or the controls. Differences in average mortality and metamorphosis were observed depending on initial developmental stage when experimentation began. In all treatment levels, high metamorphosis and low mortality occurred when exposure began during later stages of development (NF 54^+). The highest mortality in all treatments was observed at NF stage 52. Less metamorphosis occurred when exposure was started at earlier NF stages (46-50, 51 and 52) than in more advanced NF stages (53, 54 and 54^+) in all treatment levels.

1.5.3 Cellular, hormonal and genetic effects of methylmercury

Cellular, hormonal, and genetic responses to MeHg exposure have been observed using both *in vitro* cell lines and *in vivo* experimentation. For example, the *in vitro* exposure of primary cultures of rat cerebellar granule cells to MeHg by Castoldi et al. (2000) resulted in time and concentration-dependent cell death. Within 1 hour of exposure to 5-10 mM of MeHg, impairment of mitochondrial activity, de-energization of mitochondria, and plasma membrane lysis were observed, resulting in necrotic cell death. Lower concentrations of MeHg (0.5–1 mM) did not initially affect cell viability, mitochondrial membrane potential, or function, however, all cells progressively underwent apoptosis and cell death was reached by 18 hours of treatment. These results demonstrate that exposure to low levels of MeHg triggers delayed-neuronal cell death, whereas higher levels of MeHg prevent normal cell apoptosis from taking place. Therefore, in developing organisms MeHg may contribute to the *in vivo* toxicity of MeHg exposure in the developing brain.

Basu et al. (2005) found a significant positive correlation between Hg (THg and MeHg) and muscarinic cholinergic receptor density as well as Hg and ligand affinity when receptor-binding assays were conducted using brain tissue obtained from wild mink. A significant negative correlation was also found between THg and dopaminergic-2 receptor density. Disruptions to both muscarinic and dopaminergic receptors has been shown to cause behavioural changes such as an impaired ability to hunt, breed, or migrate (Caulfield and Birdsall 1998, Missale et al. 1998). Therefore, the correlations found in this study suggest that environmentally relevant concentrations of Hg may alter

neurochemical function and neurochemical receptor binding which in turn may decrease fitness.

Several studies have also investigated the hormonal effects of MeHg exposure. For example, *in vitro* research using human breast cancer cells has shown that inorganic Hg binds to the α isoform of the estrogen receptor (ER) leading to a decrease in ERα protein levels and alterations in growth-regulating signaling pathways (Martin et al. 2003). Similar results have been obtained when this cell type was exposed to MeHg in the presence of low levels of estradiol and they suggest that MeHg may have the capacity to act as an estrogen-disrupting agent (Sukocheva et al. 2005). *In vivo*, Drevnick and Sanderheinrich (2003) found that juvenile fathead minnows fed diets contaminated with MeHg have suppressed levels of testosterone in males and 17ß estradiol in females at sexual maturity. A surge of both of these hormones is required immediately prior to reproduction for successful spawning to occur. Therefore, these results may explain declines in spawning success. MeHg was also found to inhibit gonadal development in females. Reproductive success was reduced for both males and females as pairs fed MeHg either took 5 days longer than control minnows to spawn a clutch of eggs or did not spawn at all. Female fathead minnows chronically exposed to diets containing environmentally relevant concentrations of MeHg have also been shown to exhibit an increased number of apoptotic ovarian follicular cells (Drevnick et al. 2006). Ovarian follicular cells are responsible for the production of 17ß estradiol and other sex steroid hormones. Therefore, increased apoptosis of steroidogenic gonadal cells induced by MeHg exposure may suppress sex steroid hormones and ultimately impair fish reproduction.

Several studies have also investigated the effects of MeHg on the TH system. For example, *in vitro* experimentation using mouse neuroblastoma cells (Mori et al. 2006a) has shown that MeHg inhibits D2 activity in a dose and time dependant manner and that MeHg may act as an antagonist by binding competitively to its selenocysteine subunit. Inhibition of D2 halts the conversion of T_4 to the more biologically active T_3 and arrests the normal progression of development. Further research using rat pituitary tumor cells (Mori et al. 2007) has also shown that MeHg inhibits D2 activity leading to a decrease in T_4 but not T_3-induced growth hormone secretion. *In vivo*, Watanabe et al. (1999) found that the injection of pregnant mice with 3μg of MeHg per g of body weight for three days induced changes in 5'- and 5-D1, D2 and D3 activity. Increasing 5'-D1 and decreasing 5-D1 was found to cause an excess of biologically active T_3 in the brains of the fetuses which has the potential to cause hyperthyroidism and growth retardation. Another study by Mori et al. (2006b) exposed pregnant wildtype and metallothionein (MT) deficient mice to food containing 5 mg/kg of MeHg from gestational day zero to post-natal day ten. MT is a protein responsible for the detoxification of metals. At the end of this exposure offspring were examined to determine the effects of treatment on TH metabolism. Although MeHg exposure failed to induce changes in serum T_4 levels, liver D1, or brain D2 activity, a decrease in brain D3 was observed in all neonates. This suggests that the TH metabolism of fetuses and neonates may be a target of MeHg.

MeHg has also been found to disrupt gene expression and may be classified as an endocrine disrupting chemical. In a study by Klaper et al. (2006) fathead minnows exposed to environmentally relevant levels of dietary MeHg exhibited changes in the expression of genes commonly associated with endocrine disruption. Up-regulation in

liver vitellogenin mRNA in males and down-regulation in vitellogenin mRNA in female fish were observed with increasing Hg concentrations. Microarray analysis of liver tissue also identified changes in the expression of genes associated with egg fertilization and development, sugar metabolism, apoptosis, and electron transport.

Neurotoxicity arising from exposure to MeHg has been well documented in both humans and experimental animals. Most studies, however, have focused on acute or very high level exposures. The physiological, hormonal, and genetic studies described above demonstrate that low levels of MeHg can elicit sublethal effects in many different organisms. Although the changes induced by environmentally relevant levels of MeHg, or levels found in contaminated environments, may at first appear subtle or may not lead to immediate mortality within a population, they may still disrupt the normal systems governing development, growth, metabolism, and reproduction. Disruptions to these systems can have negative consequences for exposed organisms, especially those undergoing sensitive periods of development. It is therefore important to study the effects of MeHg in organisms such as amphibians that are considered to be especially sensitive indicators of environmental contamination.

Table 1.1 The effects of various chemical and physical conditions on Hg methylation in aquatic environments. Adapted from USEPA (1997).

Physical or Chemical Condition	**Qualitative Influence**
(-) dissolved oxygen	(+) methylation
(-) pH	(+) methylation in water column
(-) pH	(-) methylation in sediment
(+) DOC	(+) methylation in sediment
(+) DOC	(-) methylation in water column
(+) salinity	(-) methylation
(+) nutrient concentrations	(+) methylation
(+) selenium concentrations	(-) methylation

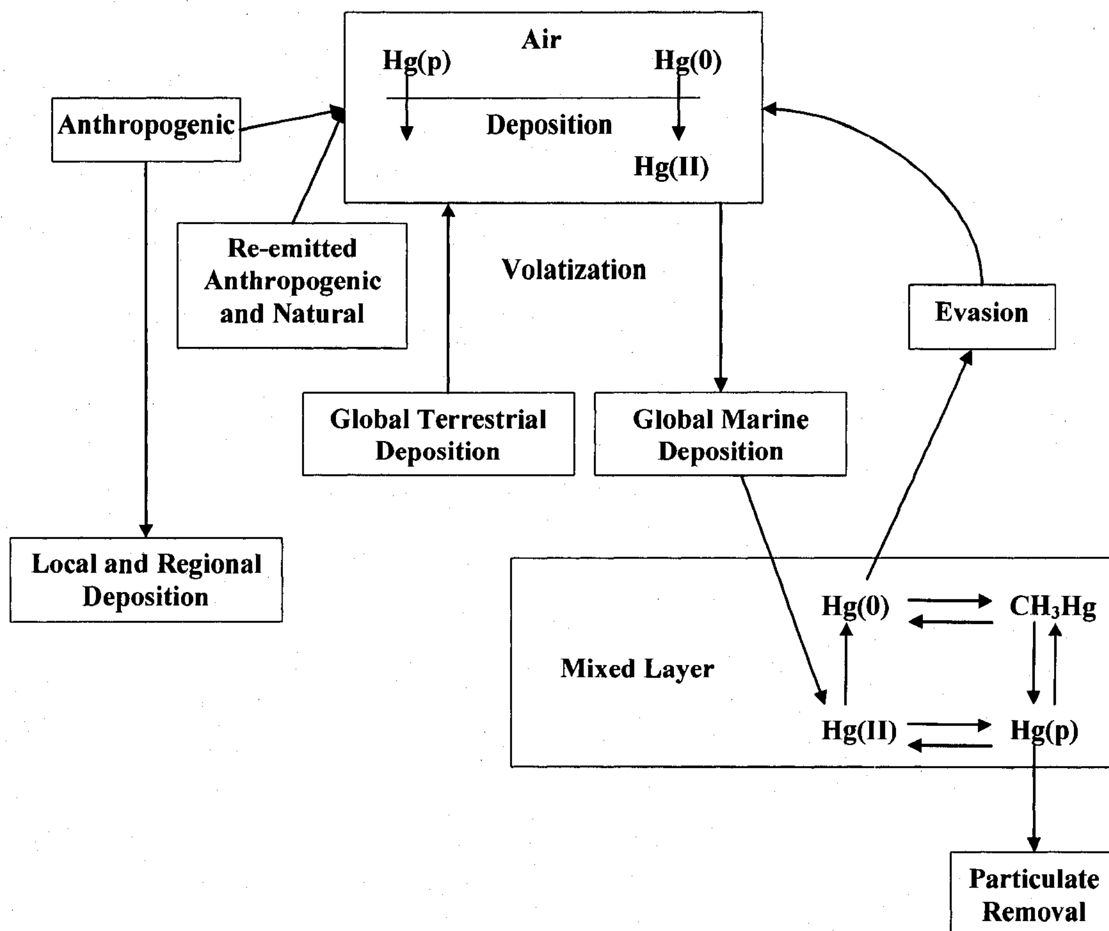


Figure 1.1 Global Hg cycle model. Hg(p) – divalent particulate Hg. Adapted from Mason et al. (1994) and Bullock (2000).

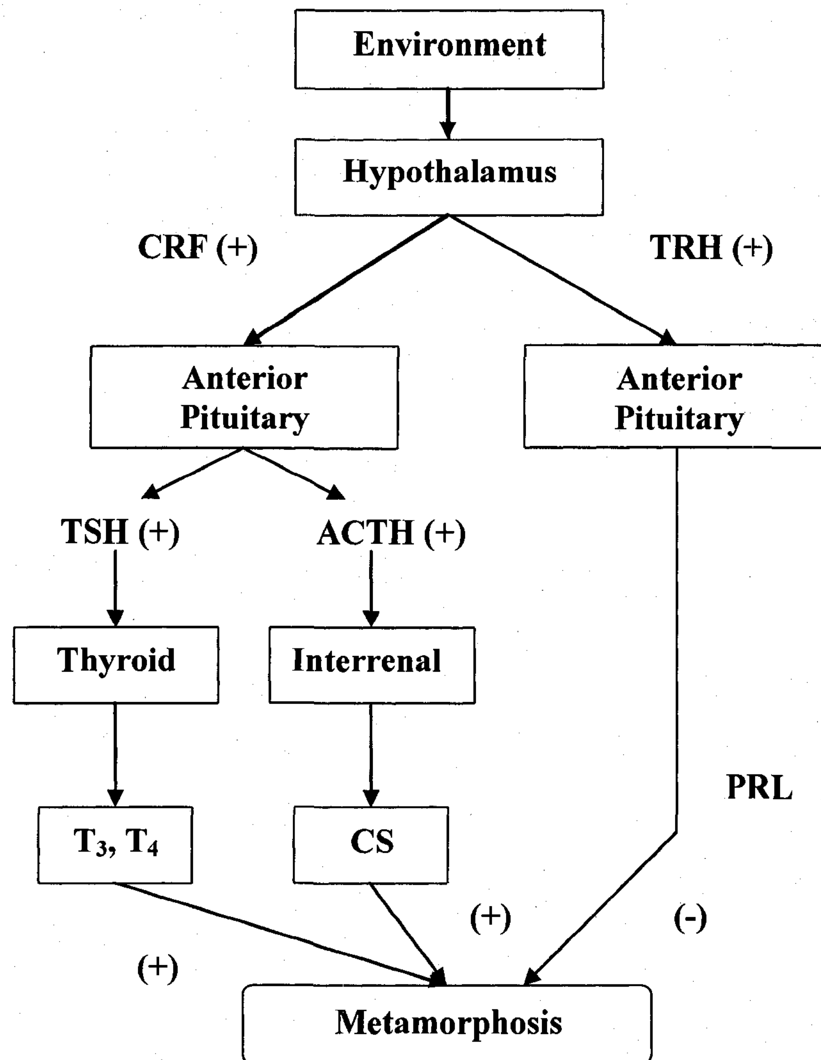


Figure 1.2. Schematic representation of the endocrine systems and hormones controlling amphibian metamorphosis. Pluses (+) designate stimulatory actions and minuses (-) designate inhibitory actions. ACTH – adrenocorticotropic hormone; CRF – corticotropin releasing factor; CS – corticosteroids; PRL – prolactin; T3 – 3-5-3' triiodothyronine; T4 – thyroxine; TRH – thyrotropin releasing hormone; TSH – thyroid stimulating hormone. Adapted from Denver (1998b) and Tata (1999).

CHAPTER 2

Fate of dietary uptake of methylmercury in *Xenopus tropicalis* tadpoles

2.1 Introduction

Most studies examining the bioaccumulation of MeHg have focused on aquatic food webs with specific concern for the top predator fish which have the highest levels of Hg. The impacts of Hg exposure on the humans that eat these fish have traditionally driven the research. Although such studies are of value, they preclude having reliable Hg budgets for organisms at other trophic levels. Instead, investigations have generally focused on the health effects resulting from specific dose rates calculated from the consumption of prey/food items with high MeHg body burdens, without information on Hg degradation or depuration pathways. Although such research is needed to identify the inherent health risks of MeHg, significant gaps in our scientific knowledge of rates of MeHg assimilation related to intake, conversion rates of MeHg to other forms of Hg, and, more importantly, how much is depurated remain unanswered. This is especially relevant to organisms inhabiting aquatic environments where they are continuously exposed to MeHg.

Studies (Unrine et al. 2004, Gibson 2005) indeed demonstrate that MeHg can have detrimental effects on amphibian tadpoles; however, few have related Hg concentrations in whole body organisms to specific exposure levels and none have explored the potential for MeHg detoxification during the sensitive periods of development and metamorphosis. The goal of this chapter is to examine patterns of MeHg exposure, bioaccumulation, and elimination in tadpoles chronically exposed to

environmentally relevant levels of dietary MeHg. Other experiments exposing tadpoles to dissolved MeHg and Hg^{2+} have been ignored as most Hg is assimilated through food intake and such exposures underestimate uptake. Here, MeHg and THg body burdens were measured in tadpoles throughout their development and patterns of accumulation and elimination were compared between low and very low treatments. Water temperature, pH, dissolved oxygen content, conductivity, hardness, dissolved organic and inorganic carbon levels, ammonia levels, and water concentrations of MeHg and THg were measured to examine potential changes in Hg speciation over time and to determine how these concentrations relate to toxicity, accumulation, and excretion.

Past research into the effects of Hg on aquatic organisms has focused on high dose aquatic exposures to fish where Hg was dissolved in the water (McKim et al. 1976, Phillips and Gregory 1979, Niimi and Kissoon 1994, Wiener and Spry 1996). It has since been shown that MeHg uptake through absorption from the water column through the gills accounts for less than 15% of accumulation and instead MeHg is accumulated primarily through dietary exposure (Hall et al. 1997). Although few studies examining the effects of Hg on amphibian embryos and tadpoles exist, most that have been conducted have also involved the addition of inorganic Hg to water. This has particular relevance as the current guideline for safe levels of Hg exposure in aquatic habitats is based on exposure to dissolved Hg (Canadian Council for Ministers of the Environment (CCME) 2006). Experiments where Hg has been dissolved in water should be ignored because they have involved the addition of greater than 0.1 μg/L of MeHg, which has been shown to cause dangerous accumulation in humans (Gaudet et al. 1995). Furthermore, the results of these experiments have led to permitted discharge from

industrial sources that far exceed the levels required for the protection of aquatic organisms and the people who consume fish from these waters (Gaudet et al. 1995). Unrine et al. (2004) demonstrated that the exposure pathway for tadpoles is more likely to be through food consumption. Therefore, experiments adding Hg to water, especially high levels may have little environmental relevance as concentrations of MeHg in natural waters are usually far lower than concentrations in the food. For example, accumulation of MeHg by algae can be at least 10^4-10^6 times greater than the concentration in the water (Ridal et al. 2006). For the present study, we exposed tadpoles to MeHg through the diet as absorption from the water column was predicted to be minimal.

The accumulation of Hg by aquatic organisms depends on several factors such as diet, life-cycle stage, tolerance/detoxification, water pH, and dissolved organic carbon levels (Boening 2000). Because atmospheric deposition of Hg leads to the accumulation and biomagnification of MeHg within aquatic ecosystems such as wetlands (Hall et al. 2008), organisms inhabiting such environments, especially areas that contain high levels of DOC, are contaminated with high levels of MeHg, or have a low pH may be more at risk for the detrimental effects of MeHg accumulation (O'Driscoll et al. 2005). Since the larvae of amphibians are fully aquatic, they are likely to be more exposed to and accumulate more MeHg throughout the entire developmental period. Most tadpoles are either herbivorous, feeding mainly on algae or periphyton, or detrivorous consuming various materials such as dead zooplankton, fish, or detritus. Some species such as *Rana sylvatica* (wood frog) or *Rana temporaria* (common frog), however, may also be predatory or cannibalistic (Sparling et al. 2000). Tadpoles that consume food sources of higher trophic levels have a higher risk of accumulating elevated levels of MeHg. Any of

the aforementioned foraging tactics, however, have the potential to cause accumulation as all food sources have the ability to bind or carry MeHg.

Relatively few studies have examined Hg accumulation in organisms undergoing embryonic development. For example in fish, most studies investigating dietary Hg accumulation have focused on adults or individuals captured in the wild. Moreover, patterns of uptake, assimilation, and excretion during development remain to be fully characterized. A recent study examining these patterns in *Oryzias latipes* (Japanese madaka) juveniles chronically exposed to MeHgCl in water, at levels comparable to typical Hg levels in polluted rivers in China, found that fish accumulated MeHg until a threshold was reached after five to six months of exposure (Liao et al. 2005). A dose-dependant increase in Hg content and a negative relationship between bio-concentration factor and exposure concentration were observed. These results, however, were not examined for growth dilution effects and patterns of excretion were not studied in juvenile fish. Adult fish exposed to the same treatment concentrations did not exhibit decreases in MeHg accumulation after eight days in clean water. It should be noted that because this experiment used water as a vector for exposure (Liao et al. 2005), it is most probable that results from dietary exposure would differ significantly because accumulation of MeHg through diet is greater.

The purpose of this study was to quantify MeHg and THg uptake, assimilation, and excretion in *Xenopus tropicalis* tadpoles exposed to environmentally relevant dietary concentrations of MeHgCl throughout development and metamorphosis. We chose the laboratory model species *X. tropicalis* to investigate MeHg accumulation because of its external development and relatively rapid life cycle. Due to a lack of information on

MeHg assimilation in tadpole larvae, our focus was on the developmental period from initial feeding until metamorphic climax. We predicted that assimilation would follow three phases: 1) initial high MeHg uptake, 2) gradual MeHg assimilation, and 3) a state of equilibrium between MeHg uptake and decontamination and/or excretion. We also predicted that we would observe a biomass dilution of MeHg concentrations due to tadpole growth over the length of the exposure, and that differences between MeHg and THg concentration in the tadpoles would be low in all treatment groups if there was little MeHg degradation, but higher if it was degraded to other forms of Hg.

2.2 **Methods**

2.21 Breeding

Xenopus tropicalis tadpoles were bred in-laboratory by giving adult frogs subcutaneous priming injections of Human Chorionic Gonadotropin (HCG) (Sigma-Aldrich Canada Ltd., Oakville, ON). Three breeding pairs were selected, injected with 50 μL of 250 unit/mL HCG and separated into 12 L tanks containing aerated University of Ottawa Aquatic Care facility water (dissolved oxygen 9.2-10 mg/L, <0.01 mg PO_4/L, <0.01 mg NO_3/L, temperature 24°C, pH adjusted to 5.8-6.0 with 1.0 N hydrochloric acid (HCl)). Approximately 24 h after the priming injection, a second injection of 250 μL HCG was administered and breeding pairs were placed in clean system water. Tanks were shaded with black plastic to prevent disturbance. All three breeding pairs began clasping within 2 h of the administration of the second injection of HCG. Once the adults unclasped, eggs were collected from each tank and randomly placed in Petri dishes containing 30 mL of 1x9 Ringer's solution and 30μL of gentamicin (Sandoz Canada Inc.,

Boucheville, QC). No more than 100 eggs were placed in each Petri dish and eggs from each breeding pair were mixed to provide genetic diversity. After hatching, all remaining eggs and dead or deformed tadpoles were removed from each dish and the 1x9 Ringer's solution was refreshed. The following day, all dead and deformed tadpoles were removed, the density of tadpoles in each dish was lowered by 10% and the 1x9 Ringer's solution and gentamicin were refreshed. This procedure was repeated for five days until all tadpoles reached Nieuwkoop and Faber (NF) stage 45/46 (Nieuwkoop and Faber 1994). All animal experimentation followed the guidelines and standards of the Animal Care Committee (University of Ottawa) and the Canadian Council on Animal Care.

2.22 Conditions of Exposure

Prior to the start of the experiment, 9.5 L aquarium tanks were acid-washed using 10% HCl and rinsed with deionized water to remove any potential traces of Hg. Each tank was filled with 5 L of University of Ottawa Aquatic Care facility water buffered to a pH of 7.5-8.0 using a 1x20 Ringer's solution, aerated (98 % dissolved oxygen saturation; Oxyguard® Hardy MK III, Point Four Systems Inc., Coquitlam, BC), and allowed to acclimatize for 24 h prior to the addition of tadpoles. Water testing before the addition of tadpoles indicated that the MeHg content of the water was below the detection limit (<0.02 ng/L) and that THg content ranged from 0.5-0.69 ng/L (approximately what is found in Ottawa River water). Approximately 100 NF stage 45/46 tadpoles were initially transferred to each tank at a density of 1-2 g/L. Tadpole numbers were reduced throughout the exposure by adding additional tanks to maintain these density conditions.

A total of 3757 tadpoles were used in this experiment, providing an average of 1252 for each of the three treatments.

A static renewal system was used for experimentation and involved the removal and replenishment of water according to a bi-weekly schedule (twice a week during the first week and once during the second week). To minimize handling stress on the animals, the water in each tank was never completely changed but was lowered by 25-40% using a gravity suction method. Feces and particulate matter were suctioned from the bottom of the tanks to prevent a buildup of organic matter. Once a week, the sides of the tanks were lightly scrubbed using a soft net and all air hoses and stones were cleaned to remove algal buildup. The water and contents noted above were sub-sampled for MeHg and THg content.

Water pH, conductivity, temperature, dissolved oxygen levels, hardness, dissolved organic and inorganic carbon levels, and ammonia levels were measured bi-weekly over the course of the experiment both before and after cleaning. Water pH ranged from 7.5-7.82, conductivity from 928-1116 µS and temperature was maintained at 25 ± 2°C (HI 98129 Combo Waterproof pH/EC/TDS/Temperature Tester, Hanna Instruments Inc., Laval, QC). Dissolved oxygen levels ranged from 90-98% saturation (Oxyguard® Hardy MK III, Point Four Systems Inc., Coquitlam, BC) and water hardness from 50-60 mg $CaCO_3$/L (Nutrafin A-7830 Test Kit Carbonate and Total Hardness, Rolf C. Hagen Inc., Montreal, QC). Dissolved organic carbon levels were between 6.99-13.53 mg/L and dissolved inorganic carbon levels ranged from 2.25-4.30 mg/L (Model 1010 Wet Oxidation Total Organic Carbon Analyzer, OI Analytical, College Station, TX). Ammonia was not detected (less than 0.6 mg/L) before cleaning or after cleaning

(Nutrafin NH3/NH4 ammonia test (0.0 – 7.3 mg/L) Rolf C. Hagen Inc., Montreal, QC). The experimental environment was controlled with a photoperiod of 12:12 light/darkness.

2.23 Experimental diets

Experimental diets consisted of food slurry spiked with MeHgCl. This diet was formulated on the principle that MeHg binds to particulate material (in this case powdered algae). To create the slurry, 1g of Sera Micron® (Sera North America Inc., Montgomeryville, PA) powdered food was mixed with deionized water in a 50 mL Falcon tube to give a food density of 0.02 g/mL. Appropriate volumes of MeHgCl (Sigma-Aldrich Canada Ltd., Oakville, ON) were added to each falcon tube to give nominal MeHg concentrations of 0, 50 and 250 ng/g MeHg wet wt. (control, low and high treatments). Falcon tubes were wrapped in aluminum foil to prevent light-induced degradation of MeHg and stored at 4°C for up to one week between feedings. Food MeHg concentrations were verified using the standard technique of gas chromatography atomic fluorescent spectroscopy (GC AFS) as described by Cai et al. (1997). Average levels were 0.42 ± 0.12, 44 ± 4 and 270 ± 13 ng MeHgCl/g Sera Micron® wet wt. for the control, low and high treatment groups respectively (1.9, 200 and 1200 ng MeHgCl/g dry wt.). Food was delivered using a 2 mL latex-free syringe, and each tank received 1mL of food daily at the beginning of the experiment. This amount was increased to 1.5 mL after 15 days of exposure and increased again to 2 mL after 40 days of exposure in order to maintain *ad libitum* feeding conditions.

MeHg delivery was tested to validate the experimental design. Clean water was dosed with tadpole food that was spiked with a known concentration of MeHgCl. After

24 h the water was filtered and analyzed for MeHg and THg concentrations. Results indicated that 85% of the MeHg was still in the particulate (>0.7μm) fraction. Therefore, it is likely that much of the remaining 15% was also bound to particulate as some small particles may have been able pass through the filter pores. In aquaria containing tadpoles, however, the Hg remaining was in the filtrate and not associated with the particles. It may represent a complex excreted by the tadpoles.

Water samples taken throughout the experiment from treatment tanks showed negligible amounts of MeHg and THg in waste water obtained 24 h after feeding. This provides evidence that most of the food was assimilated. Particulate filtered (Whatman GF/F borosilicate 47 mm diameter filter,0.7 μm particle retention, GE Healthcare, Piscataway, NJ) from waste water samples showed higher levels of MeHg within the ng/g range with recovery values of 54 ± 10, 38 ± 12 and 1.0 ± 0.09 % for the control, low and high treatments, respectively. It is important to note that the particulate was generally filtered from the water itself and not from the tadpole excrement at the bottom of the treatment tanks. The above recovery values are most likely not representative of the true values of MeHg and THg excreted by the tadpoles. Hg levels in the air were 5.0 ± 0.06 ng/m^3, which is near the normal background range for buildings (approximately 1.5-10 ng/m^3). This does not remove the possibility that some of the MeHg was converted to elemental Hg which could volatilize into the air. The volume dilution was considerable and there was consistent ventilation in the room that housed the exposure experiment.

2.24 Sampling

Tadpoles were observed daily and dead animals were removed. Tadpoles were sampled for MeHg and THg body burden analysis at distinct time intervals throughout the exposure (days 1 (0, 1, 2 and 4 hours), 2 (20 and 25 hours), 4, 8, 16, 32, 64 and 192 of exposure). At each time interval, eight samples per treatment (single or pooled) were collected (n=8). Tadpoles were euthanized using ethyl 3-aminobenzoate methanesulfonate salt (MS-222) (Sigma-Aldrich Canada Ltd., Oakville, ON) at a concentration of 1 g/L, snap frozen on dry ice and stored at -80°C until freeze drying. Freeze dried samples (Labconco Freeze Dryer 3, Labconco Corporation, Kansas City, MO) were kept at -80°C until MeHg and THg analysis. The experiment was terminated when all of the tadpoles from the high MeHg treatment group were removed by either death or sampling.

2.25 Methylmercury Analysis

MeHg water samples were collected before and after tank cleaning in pre-acid washed, distilled water rinsed, 1 L high density polyethylene (HDPE) bottles. Samples were preserved with 5 mL of American Chemical Society grade HCl and stored at 4 °C until analysis by GC-AFS was performed using the standard methods of Cai et al. (1997). The method of detection limit (MDL) was 0.02 ng/L (three standard deviations of the lowest standard (n=12) divided by the slope of the calibration). Analysis of procedural blanks consisting of de-ionized water revealed no MeHg contamination during sampling, extraction, and analysis. Analytical recovery spikes of de-ionized water (0.25 ng/L) averaged 89 ± 16% (n=22).

Tissue samples were analyzed for MeHg as described by Cai et al. (1997) with several modifications. Briefly, one to seven freeze dried tadpoles were weighed in 20 mL glass scintillation vials and 2 mL of 6N potassium hydroxide (KOH) and 2 mL of Milli-Q polished (MQ) water were added. Vials were shaken (DS-500 Orbital Shaker, VWR International, Ltd., Mississauga, ON) for 4 h at 330 rpm. After shaking, 1.6 mL of 6N HCl was added to each vial and the pH of each solution was verified and adjusted when necessary. A volume of 4 mL of 2:1 acidic potassium bromide:copper (II) sulfate ($KBr:CuSO_4$) was then added to the vials, followed by 5 ml of dichloromethane (DCM), appropriately weighed. Vials were shaken overnight at 330 rpm and centrifuged the following day at 2500 rpm for 5 min (J2-MC Centrifuge, Beckman Coulter Canada, Inc., Mississauga, ON). A portion of the DCM phase was weighed and between 3 to 4 grams was extracted and inserted into 7 mL glass scintillation vials. A volume of 1 mL of 0.01M sodium thiosulphate ($Na_2S_2O_3$) was added to each vial, the vials were shaken for 20 min at 330 rpm, vortexed for 30 s and centrifuged at 3000 rpm for 5 min. A volume of 400 μL of the $Na_2S_2O_3$ layer was extracted into 2 mL microcentrifuge tubes containing 300 μL of 2:1 acidic $KBr:CuSO_4$ solution. A volume of 300 μL of DCM was added and the tubes were shaken for 15 min, vortexed for 15 s, and centrifuged for 30 s (Eppendorf Concentrator 5301, Eppendorf Canada, Inc., Mississauga, ON). The resultant DCM bubble was extracted and passed through a pipette tip packed with dried anhydrous sodium sulphate (Na_2SO_4) into low volume glass inserts in 2 mL gas chromatography vials. Samples were kept sealed in a -20°C freezer until analysis by GC AFS. Two blanks (de-ionized water), two spikes, and two dogfish muscle standard reference material

(DORM 2- National Research Council of Canada, Ottawa, ON) vials were analyzed with each set of 24-40 sample digestions.

During GC AFS analysis (6890 Series GC System and 7683 Series Injector, Hewlett-Packard Co., San Fernando, CA), certain sample injection volumes were modified from the standard 5 μL injection to account for high MeHg concentrations in certain samples. Injection volumes of 0.2, 1 and 2 μL were used for the low (50 ng MeHg /g wet wt.) and high (250 ng MeHg /g wet wt.) treatments when necessary. A 3 μL injection volume was utilized for the 5 ng/g spikes. The MDL for tissue samples was 0.02 ng/g (three standard deviations of the lowest standard (n=12) divided by the slope of the calibration). Quality control and quality assurance samples were run during the MeHg digestions. Analysis of procedural blanks consisting of de-ionized water revealed that they were below the detection limit (<0.02ng/L) and contributed no MeHg to samples during sampling, extraction, and analysis. Analytical recovery spikes of de-ionized water (0.25ng/L) averaged 93 ± 5 % of the expected value (n=23). Average percent difference between individual samples at each time point was 19 ± 24 % which most likely represents differences in accumulation capacity between individuals.

2.26 Total Mercury Analysis

THg water samples were collected in 50 mL high density polypropylene Falcon tubes, preserved with 500 μL of bromine chloride reagent (BrCl), and stored double-bagged in Ziploc® bags at 4 °C until analysis. Water samples were analyzed for THg using dual gold trap pre-concentration and cold vapour atomic fluorescence spectroscopy (CVAFS). The analysis was conducted using a Tekran model 2600 mercury analysis

system equipped with a Tekran 2610 liquid handling module and a Tekran model 2620 autosampler module (Tekran Instruments Co., Toronto, ON). All analyses were conducted according to revised U.S. EPA Method 1631 guidelines for Hg analysis and Holmes and Lean (2006). Analysis of procedural blanks consisting of de-ionized water indicated that several sample sets were contaminated with THg. Therefore, these samples were omitted from our results. The MDL was estimated as 0.25 ng/L.

THg analysis of tissue was carried out using a SP-3D Total Hg Analyzer (Nippon Instruments Corporation, College Station, TX). Samples were combusted at 800°C and Hg was catalytically reduced to Hg^0 followed by dual gold amalgamation and detection using cold vapour atomic absorption. Freeze dried tadpoles were each placed in a clean ceramic boat prepared with the reagent powders M (1:1 $Ca(OH)_2$:Na_2CO_3) and B (Al_2O_3) in the following layered pattern: M, sample, M, B, M. The ceramic boat was wiped with a KimWipe® to remove reagent powder residue from the outside of the boat. It was placed in the combustion chamber and analyzed using mode 2 for tissue analysis, with the measurement range adjusted according to treatment groups. Blanks and check standards were run after 8 samples and a standard reference material (DORM-2) was run for every 24 samples. Quality control and quality assurance samples were run during the THg analyses. Blanks contributed an average of 0.04 ± 0.003 ng Hg to samples. Average standard reference material recovery was 100 ± 0.03 % of the expected value. Average percent difference between individual samples at each time point was 32 ± 35 % which most likely represents differences in accumulation ability between individuals.

2.27 Data analysis, calculations and statistics

Tadpole growth rates (as measured by mass gained over time) were calculated as described by New (1987):

$$\text{Mass gain (g/day)} = \text{(final mass-initial mass)/t} \qquad [1]$$

Mass balance of Hg was assessed by examining inputs, outputs and theoretical values for accumulation. Inputs of MeHg (ng/tank) were calculated by determining the amount of MeHg and THg added to each tadpole tank per day. Outputs of MeHg (ng/tank) were determined from the MeHg and THg concentrations of water removed from tadpole tanks during cleaning. Theoretical accumulation of MeHg (ng/tank) was calculated as follows:

$$\text{Theoretical} = \text{(Inputs–Outputs)/ total number of tadpoles per tank} \qquad [2]$$

Actual accumulation values were determined by multiplying the concentration of Hg in tadpoles by tadpole mass.

All statistical analyses were performed with Systat 10 (SPSS Inc., Chicago, IL). Significant differences in mass were assessed using one-way analysis of variance (ANOVA) followed by Tukey's pair-wise comparisons. Patterns of Hg accumulation were determined by fitting general linear models (GLMs) for both MeHg and THg concentrations (3-way ANOVA). Data sets were first tested for normality (Lillefore's test) and homogeneity (Levene's test) and data for mass, time, and Hg concentrations

were log-transformed to meet parametric assumptions. Hg concentrations were set as the dependant variable, treatment group as the fixed factor, and time and mass as covariates. Interactions between Hg concentrations and treatment, time, mass, treatment x time, treatment x mass, mass x time, and treatment x mass x time were assessed. Effect sizes are reported as squared multiple R (R^2). For all analyses, a level of $p \leq 0.05$ was considered to be significant.

2.3 Results

Tadpoles accumulated measurable amounts of MeHg and THg in all groups. Although initial increases in Hg concentration were followed by decreases over time, different patterns emerged when Hg concentrations were adjusted for body mass dilution. Experimentation was terminated after 192 days when 94% of the high MeHg group tadpoles had been removed from the study because of death or because metamorphic climax had been reached. The remaining tadpoles were used for body burden analysis or measurement of TH levels (see Chapter 3).

2.31 Hg concentration in tadpoles as a function of time (ng/g)

Time series for MeHg and THg tadpole concentrations in all three treatment groups are presented in Figure 2.1. In all groups, we observed an initial rapid increase in MeHg and THg concentrations. This initial rapid uptake occurred during the first week of exposure (Figure 2.2) and was followed by a significant decrease in Hg concentrations in the control group and a slower, but steady decrease in Hg concentrations in the low and high MeHg treatment groups.

In the control group, which contained very low levels of Hg due to the composition of the tadpole food, the initial period of uptake was rapid and peak concentrations of MeHg and THg were reached after 8 days of exposure (969 $\pm$ 118 and 1670 $\pm$ 62 ng/g dry wt. respectively). This was followed by a rapid decrease in Hg concentrations between days 8 and 32 and a more gradual decrease from day 32 until the final day of exposure (day 192). In the control animals, final concentrations of Hg on day 192 were 28 $\pm$ 5 and 339 $\pm$ 32 ng/g dry wt. for MeHg and THg, respectively.

The trends of MeHg accumulation in the controls were not apparent in the low and high treatments. In both MeHg exposed groups, increases in MeHg and THg concentrations were observed over time, with the accumulation phase lasting longer than that of the control group. In the low MeHg group, THg was accumulated more rapidly than MeHg (Figure 2.3). The initial rate of increase in MeHg concentration was slightly less over the first 4 days of exposure than the rate of increase between days 4 and 64 of exposure, whereas the rate of increase for THg concentration was greater over the first 4 days of exposure (Figure 2.3). This suggests that the efficiency of conversion of MeHg to inorganic Hg may be inversely related to the amount of Hg accumulated. Peak concentrations of MeHg and THg in the low MeHg group were reached after 64 days of exposure and were 12920 $\pm$ 2480 and 13710 $\pm$ 650 ng/g dry wt., respectively. In the high MeHg group, THg was initially accumulated more rapidly than MeHg. By day 64 of exposure, however, the rate of THg accumulation was less than that of MeHg accumulation (Figure 2.4). MeHg accumulation was most rapid between days 4 and 64 of exposure as seen in the low MeHg group. The rate of increase for THg concentration, however, was greatest over the first 4 days of exposure suggesting a decrease in the

conversion of MeHg to inorganic Hg over time (Figure 2.4). Peak concentrations for MeHg and THg in the high MeHg group were 103500 ± 14220 and 74110 ± 4730 ng/g dry wt., respectively.

After the 64th day of exposure, MeHg concentrations for both MeHg treatment groups exhibited decreases. MeHg concentrations on day 192 of exposure were respectively 1527 ± 124 and 30550 ± 6820 ng/g dry wt. for the low and high MeHg groups. THg concentrations in the low MeHg group decreased slightly after 64 days of exposure. THg concentrations in the high MeHg group, however, dropped sharply. It should be noted that most tadpoles exposed to the high dietary treatment did not survive until the final day of exposure. Therefore, there were an inadequate number of tadpoles for both MeHg and THg body burden analysis. The THg concentration on day 192 of exposure should be interpreted with caution as this data point represents only one tadpole tail. THg concentrations on day 192 of exposure were respectively 10530 ± 463 and 22860 ng/g dry wt. for the low and high MeHg groups.

To determine the effects of Hg treatment, exposure time, and tadpole mass on Hg concentrations, GLMs were fitted for both MeHg and THg (Table 2.1; ANOVA). Significant differences were found between the low and high MeHg groups for both MeHg and THg concentrations when compared to Hg concentrations in the control group (treatment; Table 2.1). Average Hg concentrations in tadpoles in the low MeHg group were approximately ten times higher than those in control treatment group tadpoles. Concentrations in tadpoles in the high MeHg group were six times higher than those in the low MeHg group and sixty times higher than those in the control treatment group (Figure 2.1). MeHg and THg concentrations were also found to change in relation to time

(time; Table 2.1) and tadpole mass (mass; Table 2.1). These effects remained significant for MeHg and THg when treatment groups were considered in relation to time (treatment x time; Table 2.1) and tadpole mass (treatment x mass; Table 2.1). As tadpoles were undergoing development throughout the study, tadpole mass was predicted to increase over time. Significant changes in mass over time were observed for MeHg (mass x time; Table 2.1). In contrast, these changes were not significant for THg (mass x time; Table 2.1). However, interactions of Hg concentration with treatment group and tadpole mass and time were significant for both MeHg and THg (treatment x mass x time; Table 2.1).

When comparing the mass of tadpoles used for MeHg with the mass of those used for THg analysis, mass was not significantly different within treatment groups (control groups: $F=0.518$; $df=1$; $p=0.483$; $R^2=0.04$; low groups: $F=0.329$; $df=1$; $p=0.575$; $R^2=0.02$; high groups: $F=0.325$; $df=1$; $p=0.578$; $R^2=0.02$). Tadpole mass increased in all groups over time (Figure 2.5) and tadpoles were of similar mass for the first 64 days of exposure. On the final day of exposure, however, tadpoles in the low and high MeHg groups were significantly heavier than those in the control group ($F=9.285$; $df=1$; $p=0.005$; $R^2=0.24$ and $F=9.953$; $df=1$; $p=0.004$; $R^2=0.30$, respectively). Differences in mass on the final day of exposure cannot be definitively interpreted as an effect of MeHg on development as tadpoles used for Hg analysis were sampled randomly and developmental stage was not taken into account. Average mass gain was 0.084 ± 0.01 mg/day for the control group, 0.177 ± 0.02 mg/day for the low MeHg group and 0.213 ± 0.04 for the high MeHg group.

2.32 Body burdens of MeHg and THg (ng/tadpole)

To examine changes in MeHg and THg accumulation, assimilation, and excretion at the individual level, Hg content was determined for each tadpole by multiplying the concentration (Figure 2.1) by the individual body mass. This calculation corrects for body mass dilution. Time series for MeHg and THg content per tadpole in all three treatment groups are presented in Figure 2.6. It is apparent that trends in accumulation patterns differ when adjusted for body mass. In the low and high MeHg groups, we observed an initial gradual increase in MeHg and THg content over the first 64 days of exposure, compared to a more rapid accumulation of Hg in the control group. Over the remaining 128 days of exposure, patterns of MeHg and THg accumulation differed. MeHg content reached a steady state in the control and low MeHg groups, whereas it continued to increase in the high MeHg group. THg levels continued to rise for the remainder of the exposure in all treatments.

In the control group, MeHg did not begin to accumulate until the 4th day of exposure (0.13 $\pm$ 0.03 ng/tadpole dry wt.) whereas THg began to accumulate at the onset of the experiment, albeit at relatively low levels (0.02 $\pm$ 4 x 10^{-4} ng/tadpole dry wt.; Figure 2.7). MeHg content increased gradually and the maximum concentration was reached after 64 days of exposure (0.80 $\pm$ 0.19 ng/tadpole dry wt.). A steady state was reached for MeHg content for the remainder of the exposure and content on day 192 was 0.68 $\pm$ 0.18 ng/tadpole dry wt.. THg increased for the entire duration of the experiment indicating potential elimination and depuration of MeHg. Maximum THg content was obtained on the final day of exposure (4.23 $\pm$ 0.83 ng/tadpole dry wt.).

A similar trend was seen in the low MeHg group. MeHg began to accumulate earlier, however, it did so within the first 2 hours of exposure (0.03 $\pm$ 0.001 ng/tadpole dry wt.; Figure 2.7). THg began to accumulate at the onset of the experiment, again at low levels (0.02 $\pm$ 0.002 ng/tadpole dry wt.). MeHg content increased gradually and although the maximum concentration was reached on the final day of exposure (51 $\pm$ 11 ng/tadpole dry wt.), MeHg content did not vary greatly when compared to the previous time point (64 days; 49 $\pm$ 10 ng/tadpole dry wt.) indicating a steady state between uptake and elimination. THg increased for the entire duration of the experiment once again suggesting depuration of MeHg by conversion to inorganic Hg. Maximum THg content was 402 $\pm$ 50 ng/tadpole dry wt. (day 192).

In the high MeHg group, MeHg content increased within an hour of the first feeding (0.12 $\pm$ 0.01 ng/tadpole dry wt.; Figure 2.7) and no steady state was reached as MeHg accumulation continued to increase for the duration of the study. A maximum MeHg content of 937 $\pm$ 111 ng/tadpole dry wt. was reached on the final day of exposure. THg content increased gradually until day 64 of exposure (388 $\pm$ 87 ng/tadpole dry wt.) after which it increased sharply. Maximum THg concentration on day 192 was 5390 ng/tadpole dry wt. which mostly likely underestimates the actual THg content at this time point since it represents only one tadpole tail.

On average, MeHg content per tadpole was less than THg content (Figure 2.6). When comparing percentages of MeHg during the initial accumulation phase (day 4) with percentages on the final day of exposure (day 192; Figure 2.8), percentage of MeHg within THg body burden was significantly less on the final day of exposure for the low and high MeHg groups, and slightly less for the control group. Different tadpoles were

used for MeHg and THg analysis at each time point. Therefore, these data can only be analyzed qualitatively. These trends, however, may be indicative of depuration of MeHg. The percentage of MeHg within THg for the high treatment on the final day of exposure may underestimate the true value as THg content reflects only one tadpole tail.

2.33 Water concentrations

Filtered and unfiltered water samples from all tanks were analyzed for MeHg and THg to determine patterns of speciation in the water between days 20 and 60 of exposure and to compare these patterns to the amount of Hg ingested per tadpole and by all tadpoles per day. Initial Hg concentrations in the buffered Aquatic Care Facility water were <0.02 ng/L for MeHg and ranged from 0.50 to 0.69 ng/L for THg. During the exposure experiment, MeHg concentrations in the water averaged 0.179 ± 0.02 ng/L for control tanks, 2.74 ± 0.41 ng/L for the low MeHg tanks, and 3.49 ± 0.22 ng/L for the high MeHg tanks. Water concentrations were measured during tank cleaning and water samples were always taken 24 hours after the previous feeding when the water had been fully cleared of food by the tadpoles. Sampling concentrated on the water itself and not on filtering detritus or tadpole excrement.

For all treatments, average THg concentrations were higher than MeHg concentrations in water throughout the sampling period (Figure 2.9). THg concentrations increased with increasing dose of MeHg; the control group had the lowest concentrations of Hg whereas the high MeHg group had the highest concentrations. In the control group (Figure 2.9), a slight decrease in MeHg concentration occurred over time (day 21: 0.170 $\pm$ 0.02 ng/L; day 58: 0.018 $\pm$ 0.08 ng/L; R^2=0.83) whereas THg concentration remained

relatively stable (day 21: 1.11 $\pm$ 0.02 ng/L; day 58: 0.730 $\pm$ 0.03 ng/L; R^2=0.16). The concentration of MeHg was only 14% of that of THg and because there was a slight decrease in MeHg, it may be indicative of the elimination of MeHg as inorganic Hg. In the low MeHg group (Figure 2.9), MeHg concentration did not change significantly over time (day 21: 2.89 $\pm$ 0.19 ng/L; day 53: 3.36 $\pm$ 0.06 ng/L; R^2=0.03). Unlike in the control group, THg concentration did increase slightly (day 21: 11.9 $\pm$ 0.29 ng/L; day 58: 13.9 $\pm$ 0.84 ng/L; R^2=0.84). The concentration of MeHg in the low MeHg group tanks was 24% of that of THg, potentially indicating excretion of MeHg by low treatment tadpoles. Because THg concentration increased slightly over time, this could also indicate some elimination of MeHg as inorganic Hg. In the high MeHg group (Figure 2.9), MeHg concentration decreased over time (day 21: 3.91 $\pm$ 0.10 ng/L; day 58: 2.55 $\pm$ 0.84 ng/L; R^2=0.84) whereas THg concentration increased significantly (day 25: 29.6 $\pm$ 3.0 ng/L; day 58: 51.1 $\pm$ 2.2 ng/L; R^2=0.98). The percentage of MeHg in the water relative to THg was only 9% which implies increased uptake of MeHg by high treatment tadpoles, as well as some elimination as inorganic Hg. Based on the above concentrations of MeHg and THg in the tank water of all groups, and on tank density (1-2 g/L), the amount of Hg ingested by tadpoles and for all of the tadpoles in the tanks per day was calculated. Tadpoles were found to have ingested respectively 0.035, 7.5 and 52 ng Hg/g dry wt./day for the control, low and high MeHg groups. Expected Hg content on the final day of exposure was 13, 2700 and 19000 ng Hg/g tadpole dry wt. for the control, low and high groups, respectively. As measured values in this experiment were less than expected values (Figure 2.6), it is possible that MeHg was demethylated and lost, or that Hg was excreted in a complex by the tadpoles and was not measured.

2.34 Mass balance

Based on the amount of Hg added to tadpole tanks each day, the amount removed through tank cleaning, and the density of tadpoles in each tank, Hg mass balances were calculated. In all treatments (Figures 2.10-2.12), inputs were higher than outputs and the differences between both were expected to equal accumulation. This was not the case. Actual measured values for MeHg and THg in all groups closely approximated theoretical values and were much lower than those for inputs. Therefore, approximately 95% of Hg within the system could not be unaccounted for. This Hg was most likely excreted by the tadpoles and was subsequently removed through cleaning. As Hg concentrations in tadpole excrement were not measured during water analysis, elimination through excretion is not fully accounted for and is clearly required.

2.4 Discussion

To my knowledge, this is the first study to characterize Hg dynamics in amphibians undergoing development. Over the course of the exposure experiment, tadpole body burdens (ng/g) for MeHg and THg in all groups increased over time. A steady state, however, was not reached. Instead, Hg levels increased until a maximum body burden was reached, and then decreased over time for the remainder of the study in all groups. Initial uptake during the first days of exposure was rapid for all groups. This is consistent with what is observed in fish exposed to dietary MeHg. In fish, MeHg is solubilized and rapidly transferred from the intestine to blood in a matter of hours and transferred to the visceral organs within a matter of days leading to rapid initial increases in concentration (Leaner and Mason 2004). The control group reached its maximum

concentrations of both MeHg and THg after 8 days of exposure, whereas the low and high MeHg groups accumulated Hg for longer, reaching their maximum concentrations at 64 days of exposure. This suggests that tadpoles exposed to very low levels may have a greater capacity for MeHg demethylation/detoxification and that elimination may occur more rapidly when compared to the elimination capacity of tadpoles exposed to higher levels of MeHg.

After maximum concentrations of MeHg and THg were reached, both MeHg and THg levels in all groups decreased rapidly until the final day of exposure. We predicted that MeHg levels would reach a steady over time, assuming that no demethylation and depuration was occurring, especially in tadpoles exposed to low levels of MeHg. However, significant decreases in MeHg body burdens were observed that were not expected since MeHg is readily absorbed and inefficiently removed from the bodies of vertebrates (Clarkson, 1994). If decreases in MeHg concentrations were to occur because of demethylation, they would have been accompanied by increases in THg levels. This was not the case as THg levels also decreased over time in all groups.

Results of the General Linear Model analysis indicated that Hg concentration was dependant on treatment, time and body mass. Therefore, changes in the mass of a developing organism exposed to Hg over time should not be overlooked. As expected, both MeHg and THg concentrations increased with increasing Hg dose and concentrations in each treatment group changed over time. Hg concentrations were dependant on tadpole mass as tadpoles were undergoing development over the course of the exposure and increased in size. Tadpole mass on the final day of exposure differed significantly between MeHg treatment groups and the control group; tadpoles in the

MeHg treatments groups were heavier than those in the control group. Tadpoles of numerous species have been shown to possess phenotypic plasticity, that is to say the ability to accelerate or retard growth in response to environmental stressors (Denver 1997). This led me to surmise that the Hg burden would result in differences in growth rate and/or body mass. Increased tadpole mass relative to controls implies a developmental delay because of treatment (Crespi and Denver 2005). These observed differences in the Hg treated groups, however, are inconsistent with differences in the mass of tadpoles sampled at metamorphic climax (NF stage 60) for TH analysis (Chapter 3). Metamorphic tadpoles in the high MeHg group were larger and heavier at climax than those in the control or low groups. The smaller size of the control group tadpoles, however, could perhaps explain why Hg processing was much faster in these tadpoles. In fish, the metabolic rate per unit body mass of a small fish is generally much higher than that of a larger fish. Therefore, the rate of food digestion per unit body mass, and consequently MeHg processing, is faster in smaller fish (Gerald 1973). It is unknown if the rate of Hg processing was faster because of an increased metabolism or because Hg is cleared more rapidly by depuration/detoxification mechanisms at very low levels.

When MeHg and THg concentrations were quantified as ng/tadpole, different patterns of Hg accumulation became apparent. In all groups, a rapid initial uptake of MeHg and THg occurred during the first two weeks of exposure. This was followed by a state of equilibrium for MeHg in the control and low MeHg groups where additional MeHg was mostly likely demethylated (as reflected in further increases in THg content for the remainder of the exposure). The same trend was not observed in the high MeHg group, as MeHg and THg levels continued to rise and never reached a state of

equilibrium. This is consistent with what has been observed in other experiments as growth has the capacity to reduce MeHg concentrations significantly in aquatic organisms by causing a greater than proportional gain in biomass relative to MeHg (Karimi et al. 2007).

The high proportion of MeHg to THg for the low and high MeHg groups during the first month of exposure is due to the rapid and preferential uptake of MeHg by the gastrointestinal tract (Clarkson et al. 2007). While inorganic mercury is more abundant in natural ecosystems, it is absorbed less efficiently than MeHg, and if taken up, is eliminated more rapidly (Huckabee et al. 1979, Boudou and Ribeyre 1985, Trudel and Rasmussen 1997). In fish, rates of MeHg excretion are much slower than rates of uptake (Trudel and Rasmussen 1997) and we have observed similar trends in amphibian larvae. MeHg is not eliminated quickly enough to prevent accumulation. Therefore, a steady state is maintained or if Hg levels greatly exceed elimination, levels continue to rise. The proportion of MeHg within THg body burdens was initially lower for the control group than for MeHg treatment groups. The percentage of MeHg, however, decreased in all treatment groups over the course of the exposure experiment. Decreases in the proportions of MeHg over time correspond to patterns observed in fish where assimilation decreases with increased exposure time (Houck and Chech 2004).

The assimilation efficiency of MeHg is generally dose dependant, with more efficient MeHg assimilation occurring at lower doses (Houck and Chech 2004). Therefore, the control group was predicted to have a higher proportion of MeHg to THg early in the exposure, which was not observed in this experiment. In general, bioaccumulation has been found to decrease with increasing metal concentrations for

most organisms (McGeer et al. 2003). There are, however, certain exceptions to this rule. For example, cyprinid fish exposed to Hg^{2+} and centrarchid fish exposed to MeHg do not show significant relationships between bioaccumulation and concentration (McGeer et al. 2003). The lower proportion of MeHg in the control group suggests that other factors may be involved in detoxifying/eliminating MeHg and again, that these factors may be more efficient at clearing lower levels of MeHg. Therefore, our findings suggest that the key to differences in patterns of MeHg accumulation in *X. tropicalis* tadpoles may not be in the ability to accumulate MeHg, but in the ability to eliminate MeHg.

Originally, we predicted that decreases in MeHg content in tadpoles would be due to demethylation and that this would be reflected by a difference in the rate of increase in THg versus MeHg in the organism. Excretion of Hg would be reflected by increases in Hg concentration in the surrounding medium. Analysis of tank water for MeHg and THg for each group revealed that differences in concentrations existed between treatments. MeHg concentrations decreased in the control and high MeHg groups whereas they remained stable over time in the low MeHg group. THg concentrations decreased in the low and high MeHg groups but remained relatively stable in the control treatment group. Water sampling occurred before tadpole MeHg content per tadpole reached a steady state in the control and low MeHg groups. Therefore, changes in MeHg and THg concentration over time may not be fully characterized. Also, because Hg levels in detritus and excrement were not measured a complete picture of elimination is not available. Future experiments should ensure that water sampling occurs for the duration of the experiment to determine Hg concentrations during steady state and elimination phases. They should also measure Hg concentrations in tadpole excrement as the majority

of the MeHg consumed by the tadpoles most likely passed through them directly or was assimilated and then depurated through fecal excretion. Sampling should focus on the detritus that settles on the bottom of the tadpole tanks as this is most likely the place where the MeHg has been lost.

Bioaccumulation of Hg by organisms is dependant on both assimilation and depuration processes. The biochemistry of absorption and excretion, however, is highly complex. In tadpoles, Hg is primarily absorbed by the gastrointestinal tract (and to a much lesser extent through the skin) and Wolfe et al. (1998) demonstrated that approximately 95% of an ingested dose of MeHg is absorbed into the blood stream directly from the gastrointestinal tract, whereas inorganic Hg is less efficiently absorbed (7-15%). Once absorbed by the gastrointestinal tract, MeHg is bound to proteins that allow it to cross the intestinal membrane by mimicking the structure of the amino acid methionine (Clarkson et al. 2007). Once across the intestinal membrane, bound MeHg enters the bloodstream and is distributed to all tissues in the body of the exposed organism.

MeHg accumulates in the endothelial cells of the liver in both mammals and amphibians (Loumbourdis and Danscher 2003). In adult liver cells, MeHg complexes are secreted in bile as a complex with the tripeptide glutathione on endogenous carriers and are reabsorbed by the gastrointestinal tract for excretion in feces (Clarkson et al. 1981, Ballatori and Clarkson 1985). Exposure to organic Hg results primarily in excretion via the fecal route, whereas exposure to inorganic Hg results in renal excretion (Zalups 2000). Neonatal mammals do not secrete glutathionine into bile and they do not secrete MeHg. Instead, in the liver MeHg-glutathionine complexes are hydrolyzed and released

as amino acids and MeHg-cystein complexes. These complexes are then released back into the bloodstream where they are reabsorbed by the gallbladder limiting the amount of MeHg that is absorbed by the gastrointestinal tract, and ultimately limiting MeHg excretion from the body (Clarkson et al. 2007). It is unknown if similar excretion mechanisms exist in developing amphibians and should serve as the basis for further study.

The steady states of Hg that were observed in the control and low treatment groups suggest that tadpoles exposed to low, environmentally relevant levels of MeHg have the potential to demethylate or selectively excrete it. As little is known about MeHg detoxification and excretion in amphibians, more research is required to determine the mechanisms involved. It is known that ten species of amphibians possess at least one isoform of metallothionein (MT), a metal-detoxification protein known to bind and eliminate Hg in mammals (Suzuki et al. 1983, Suzuki and Akitomi 1983, Suzuki and Tanaka 1983, Yamamura and Suzuki 1983, Suzuki et al., 1984, Suzuki and Kawamura 1984, Suzuki et al. 1986, Suzuki and Ebihara 1984, Saint-Jacques et al. 1998 and Dobrovoljc et al. 2003). MTs are ubiquitous within the body and exposure to Hg induces their synthesis. These proteins have an extremely high content of cysteine residues which allows them to bind to metal centers and enables them to serve as a heavy metal-detoxification system (Leiva-Presa et al. 2004). Although the binding of Hg^{2+} to MTs has been extensively studied, the formation of MeHg-MT complexes has attracted much less attention. Early studies found that MTs had no significant role in the detoxification of MeHg as they were unable to bind to MeHg either *in vivo* or *in vitro* (Leiva-Presa et al. 2004). New evidence, however, demonstrates that MTs can in fact bind MeHg and that

induction of MTs in the brain can detoxify MeHg and even reverse cytotoxicity (Hidalgo et al. 2001). It is unknown if there is a threshold level for MeHg accumulation that if surpassed, overwhelms the detoxification ability of MTs. If this is the case, it may explain the lack of MeHg detoxification in the high MeHg group of this experiment. More research into the chemical reactions between MTs is warranted, especially in non-mammalian vertebrates as little information exists.

As previously discussed in Chapter 1, environmental Hg concentrations in tadpole diet items can vary depending on the item and the location of sampling. For example, levels in pristine environments free of point sources of Hg can range from 10 to 20 ng MeHg/g dry wt. in suspended particulate matter (Plourde et al. 1997), and up to 1370 ng/g dry wt. MeHg in large fish (Sarica et al. 2005). Levels in aquatic environments that are contaminated, such as those receiving Hg-contaminated effluent from industry, can range from 2700 ng MeHg/g dry wt. in suspended particulate matter (Wilken and Hintelmann 1991), to 50000 ng THg/g dry wt. in periphyton and nearly 250 ng MeHg/g wet wt. in grazing fish (Hill et al. 1996). Therefore, the MeHg concentrations for the control and 50 ng/g wet wt. diets (1.9 and 200 ng/g dry wt. respectively) in the present exposure experiment can be considered environmentally relevant. In relation to the aforementioned concentrations of Hg, the 250 ng/g wet wt. diet (1200 ng/g dry wt.) used in our study is representative of concentrations found in diet items of higher trophic position in unpolluted environments and diet items of lower trophic position in Hg contaminated environments. It should be noted that the MeHg present in the control diet is due to the composition of the tadpole food (Sera Micron®) as it contains ground up algae and fish that also naturally accumulate MeHg. This food is a standard diet for *X.*

tropicalis tadpoles (Grainger Lab 2001) and has been used at the University of Ottawa Aquatic Care Facility for a number of years. It has not been found to have deleterious effects on tadpole growth or behaviour (personal observations; also see Chapter 3).

The results of this study demonstrate that different patterns of MeHg accumulation exist in *X. tropicalis* tadpoles exposed to low and high concentrations of dietary MeHg. Our original predictions that 1) initial high MeHg uptake would occur, 2) this would be followed by more gradual MeHg assimilation as MeHg is converted to other Hg forms, and 3) this would lead to either a steady state where MeHg uptake and degradation and/or excretion would occur, held true when tadpoles were exposed to low levels of MeHg. The steady states observed were accompanied by increases in THg content in the tadpoles, suggesting that excess inputs of MeHg were demethylated and released as inorganic Hg. In the high MeHg group, the rate of accumulation did decline but no steady state was reached and Hg continued to accumulate over time. Initial MeHg and THg uptake was rapid, eventually giving way to more gradual increases in MeHg content and sharp increases in THg content. Although the mechanisms governing MeHg detoxification in tadpoles remains to be elucidated, this is the first study to show that detoxification by developing amphibians is possible when levels are representative of those found in pristine environments. However, the present study also demonstrates that higher levels of MeHg, such as those found in organisms of higher trophic levels in pristine environments or organisms of lower trophic levels in moderately Hg polluted environments, appear to have the capacity to override whatever protective physiological mechanisms are in place. Further research into detoxification mechanisms and more

rigorous measurement of Hg concentrations in water and detritus/excrement are necessary to fully characterize these phenomena.

Table 2.1. Results of general linear models for Hg concentrations fitted for MeHg and THg. Treatment (control, 50 ng/g and 250 ng/g) was the fixed factor, time (days) and tadpole mass (g) were covariates (five fitted coefficients).

Source	MeHg				THg			
	df	F-ratio	p	R^2 [a]	df	F-ratio	p	R^2
Treatment	2	10.17	<0.001	0.86	2	14.69	<0.001	0.94
Time	1	4.560	0.034		1	99.25	<0.001	
Mass	1	5.871	0.016		1	77.53	<0.001	
Treatment x Time	2	5.260	0.006		2	36.39	<0.001	
Treatment x Mass	2	7.566	0.001		2	18.69	<0.001	
Mass x Time	1	21.45	<0.001		1	0.008	NS[b]	
Treatment x Mass x Time	2	5.450	0.005		2	19.04	<0.001	
Error	276				263			

[a]R^2 denotes effect size
[b]Not significant

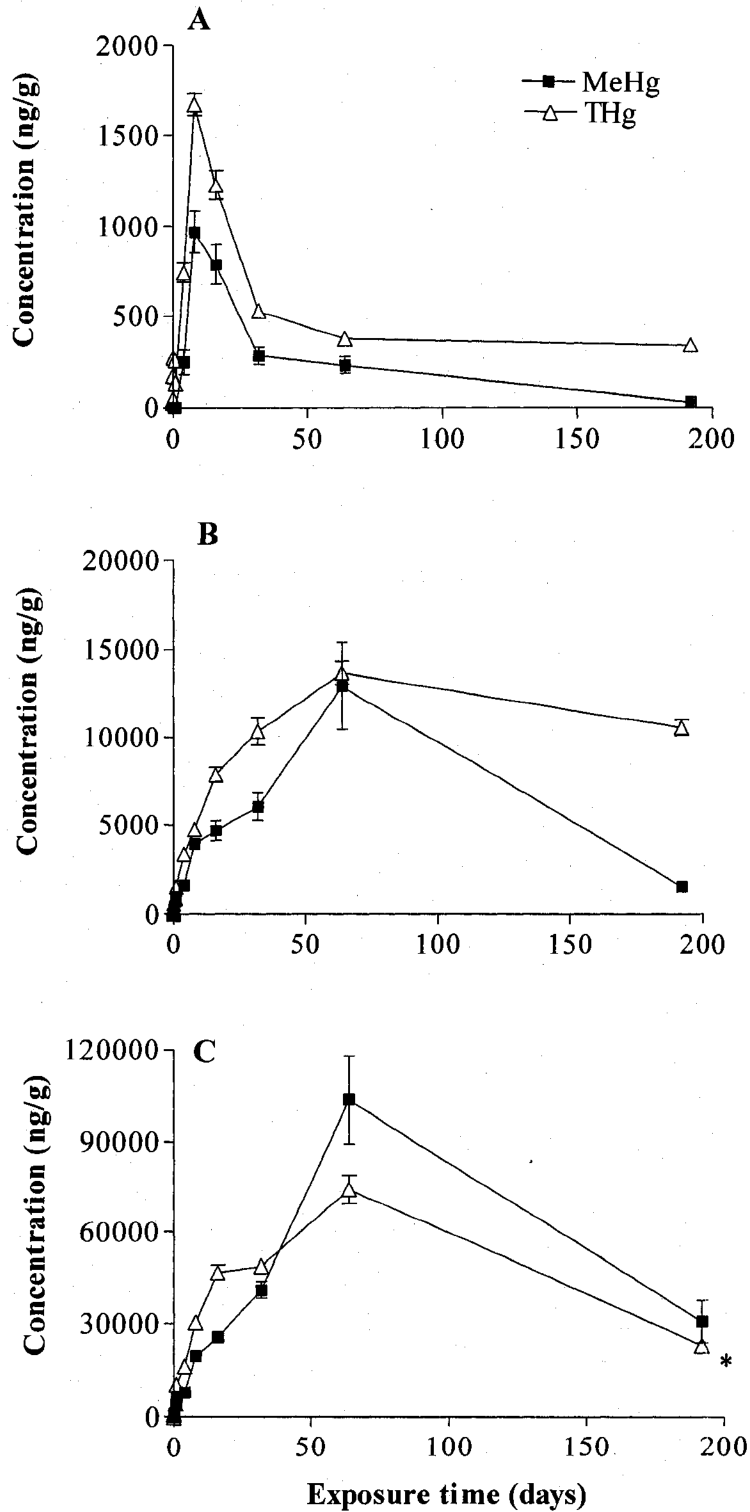


Figure 2.1. MeHg and THg concentration as a function of time in tadpoles from the control (**A**), 50 ng/g (**B**) and 250 ng/g (**C**) treatment groups ($\pm$ SE). Note different scales on the y axes. $n \geq 6$. *THg concentration on the last day of exposure for the 250 ng/g treatment group reflects only one tail.

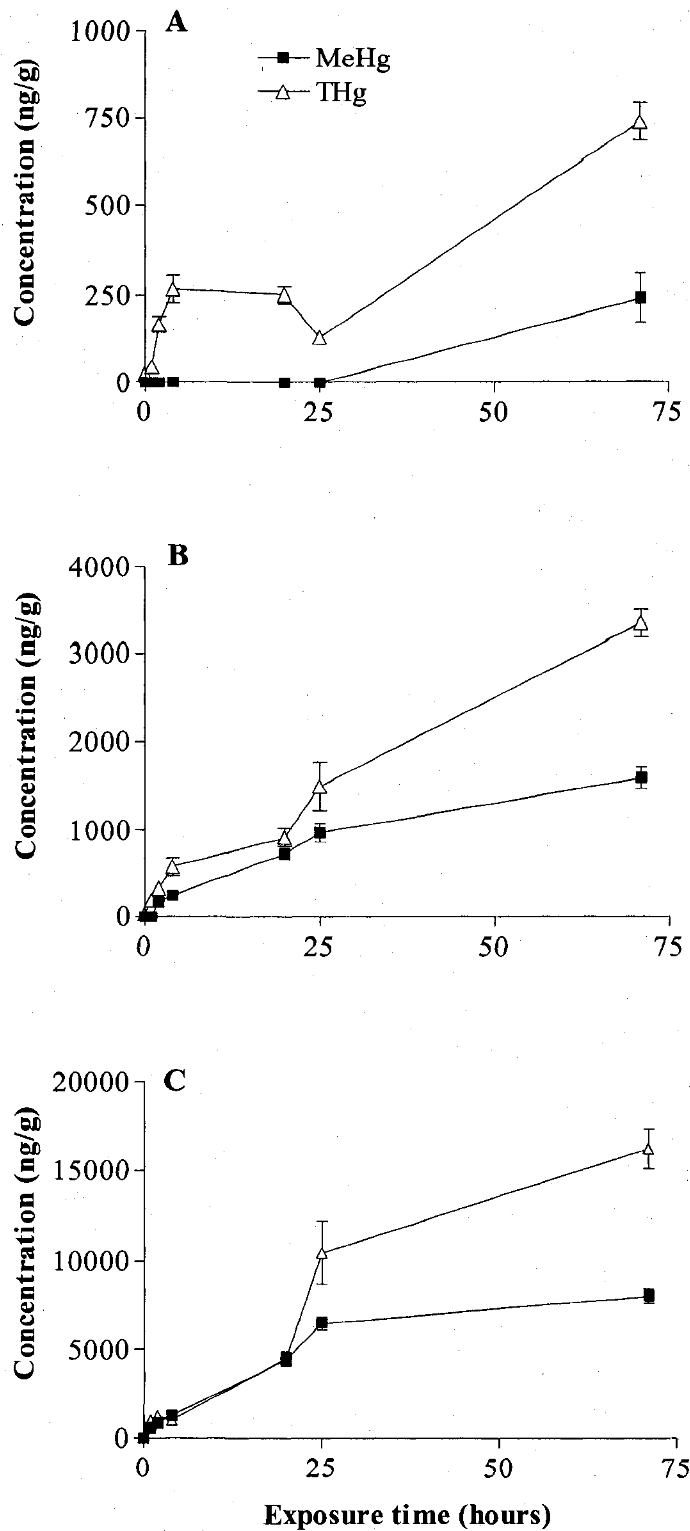


Figure 2.2. MeHg and THg concentration over the first 4 days of exposure in the control (**A**), 50 ng/g (**B**) and 250 ng/g (**C**) treatment groups ($\pm$ SE). Time is expressed in hours with day 1= 0, 1, 2 and 4 hours; day 2= 20 and 25 hours; day 4=71 hours. Note different scales on the y axes. n$\geq$6.

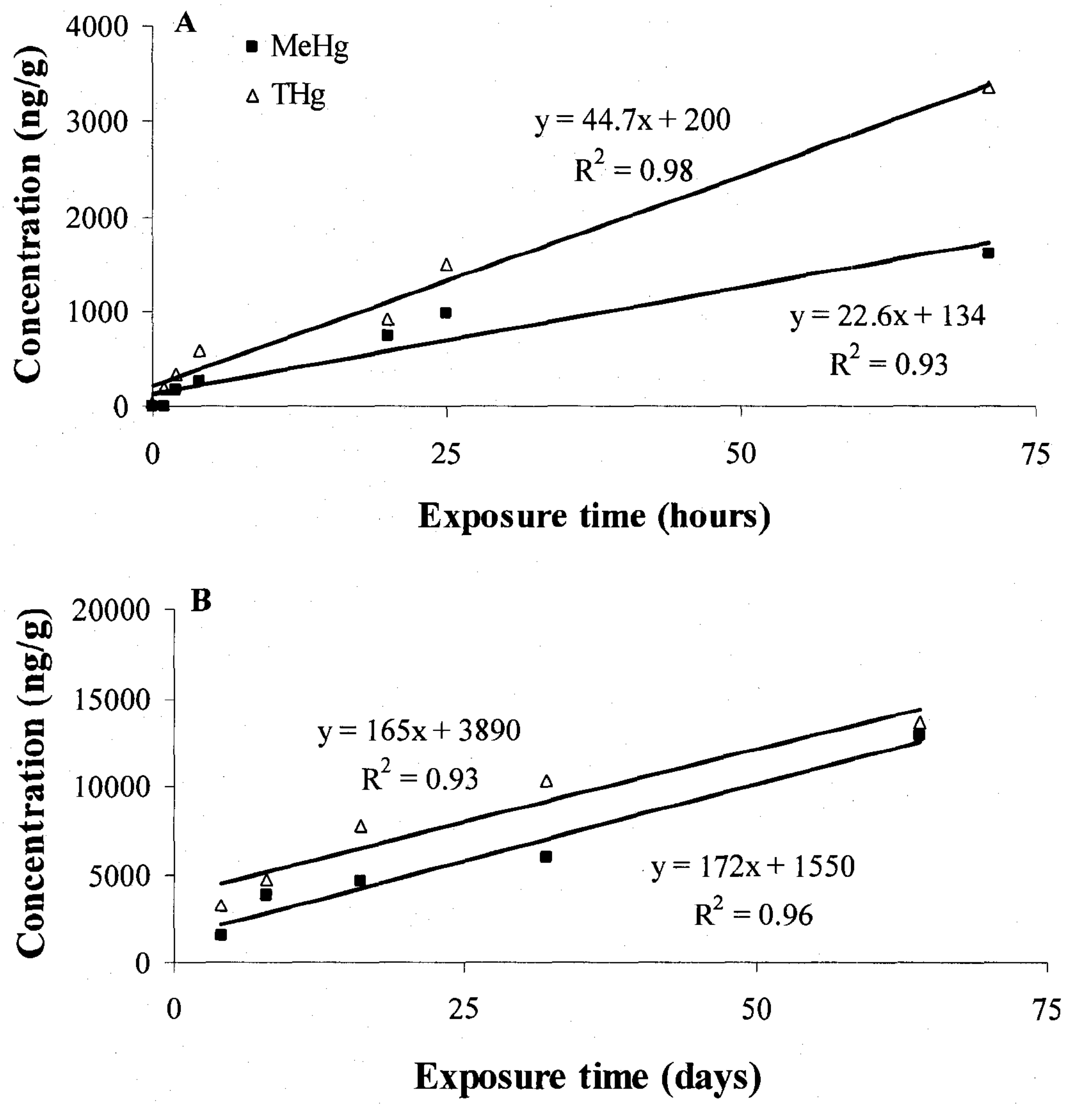
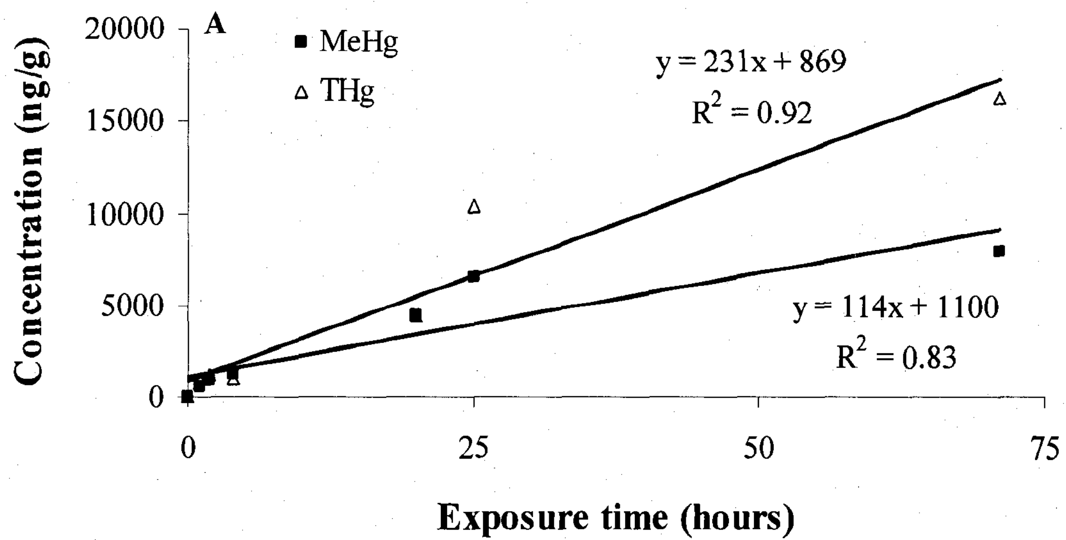


Figure 2.3. MeHg and THg accumulation in the 50 ng/g treatment group over (**A**) the first 4 days of exposure and (**B**) days 4 to 64 of exposure. Note different scales on the y axes. n≥6.



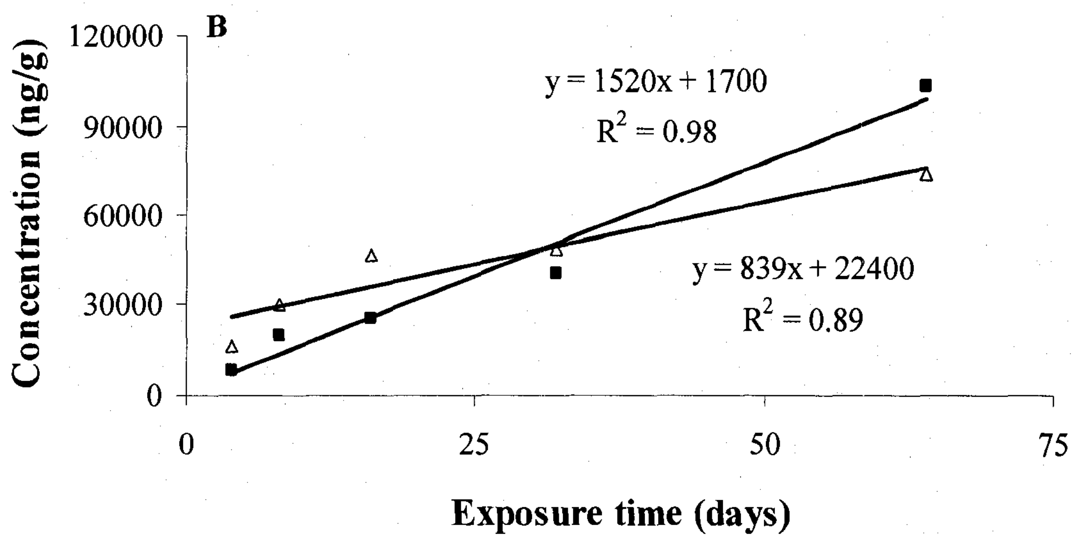
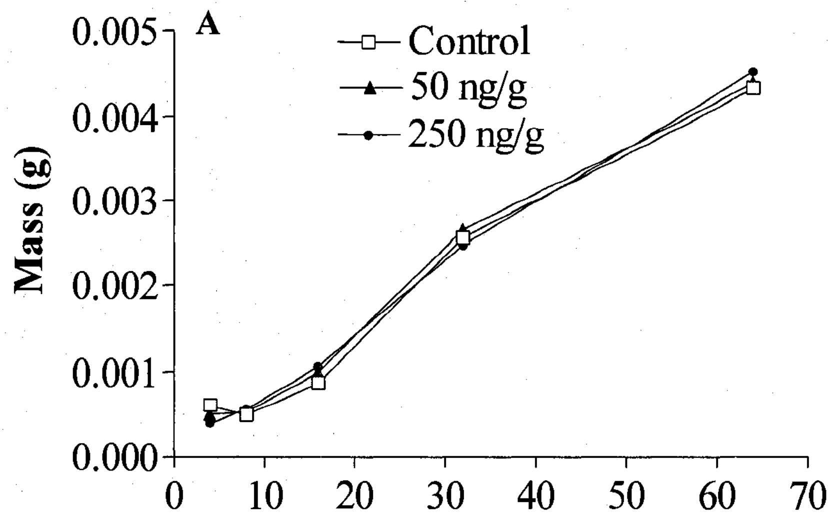


Figure 2.4. MeHg and THg accumulation in the 250 ng/g treatment group over (**A**) the first 4 days of exposure and (**B**) days 4 to 64 of exposure. Note different scales on the y axes. n≥6.



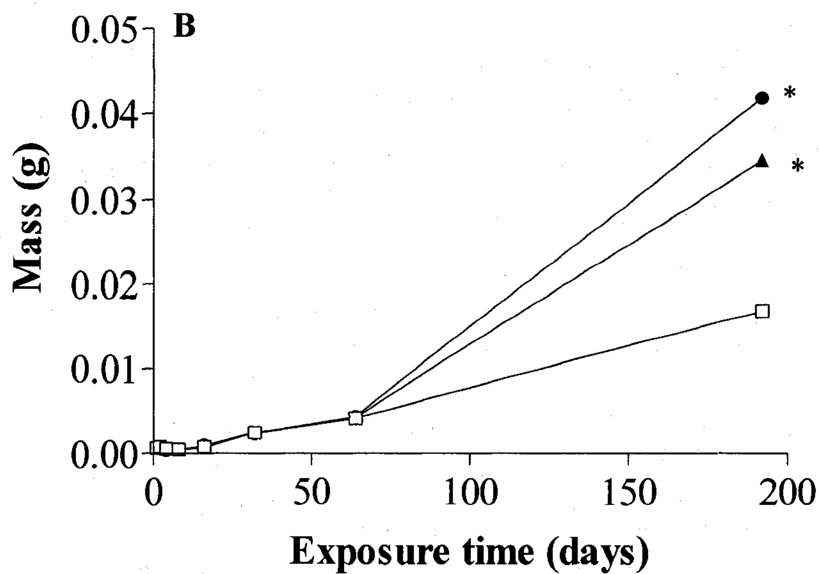


Figure 2.5. Growth of tadpoles during (**A**) the first 70 days of exposure and (**B**) the entire exposure time. Data were assessed by one-way ANOVA followed by Tukey's pair-wise comparisons (significance at $p \leq 0.05$). Asterisk (*) indicates a significant difference when compared to the control group ($p=0.002$). Note different scales on the x and y axes.

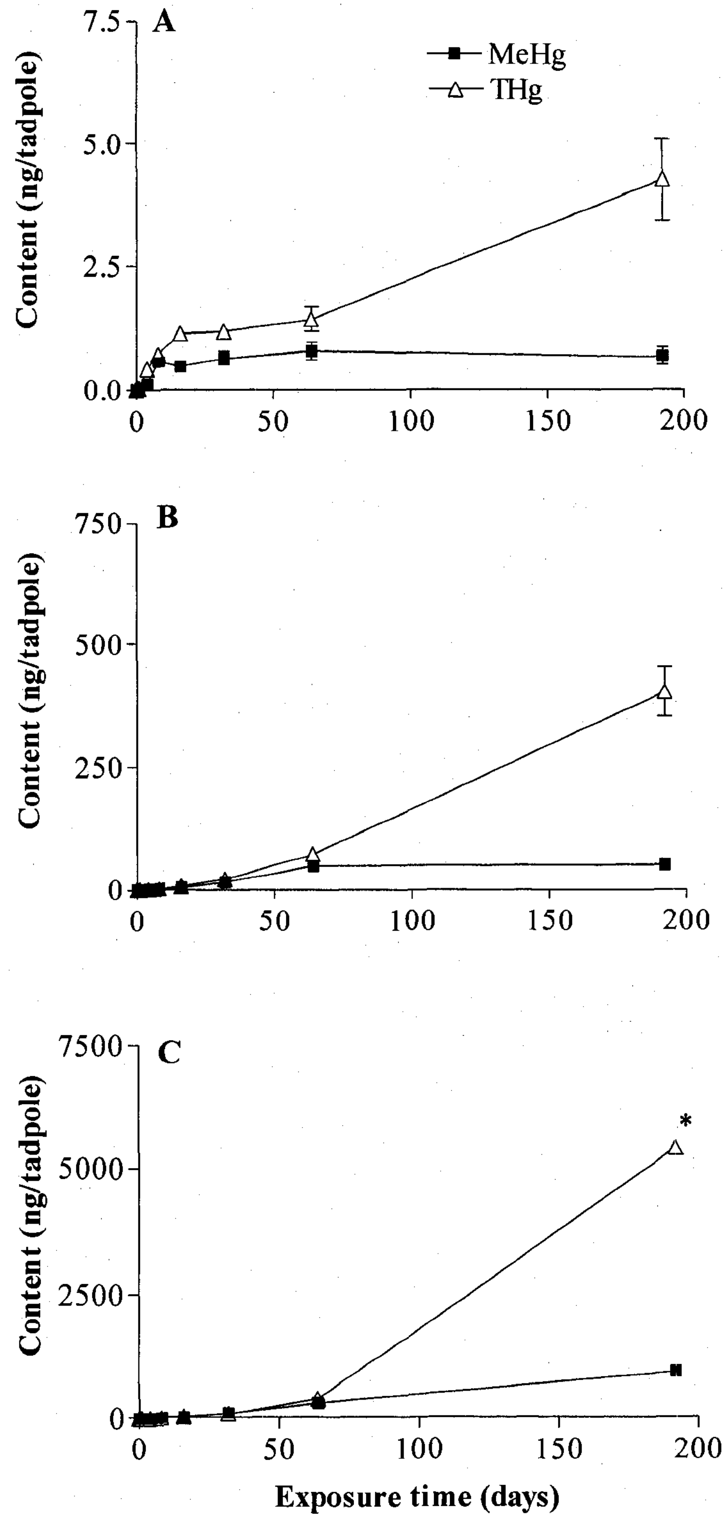


Figure 2.6. MeHg and THg content per tadpole as a function of time in the control (**A**), 50 ng/g (**B**) and 250 ng/g (**C**) treatment groups ($\pm$ SE). Note different scales on the y axes. n≥6. *THg concentration on the last day of exposure for the 250 ng/g treatment group reflects only one tail.

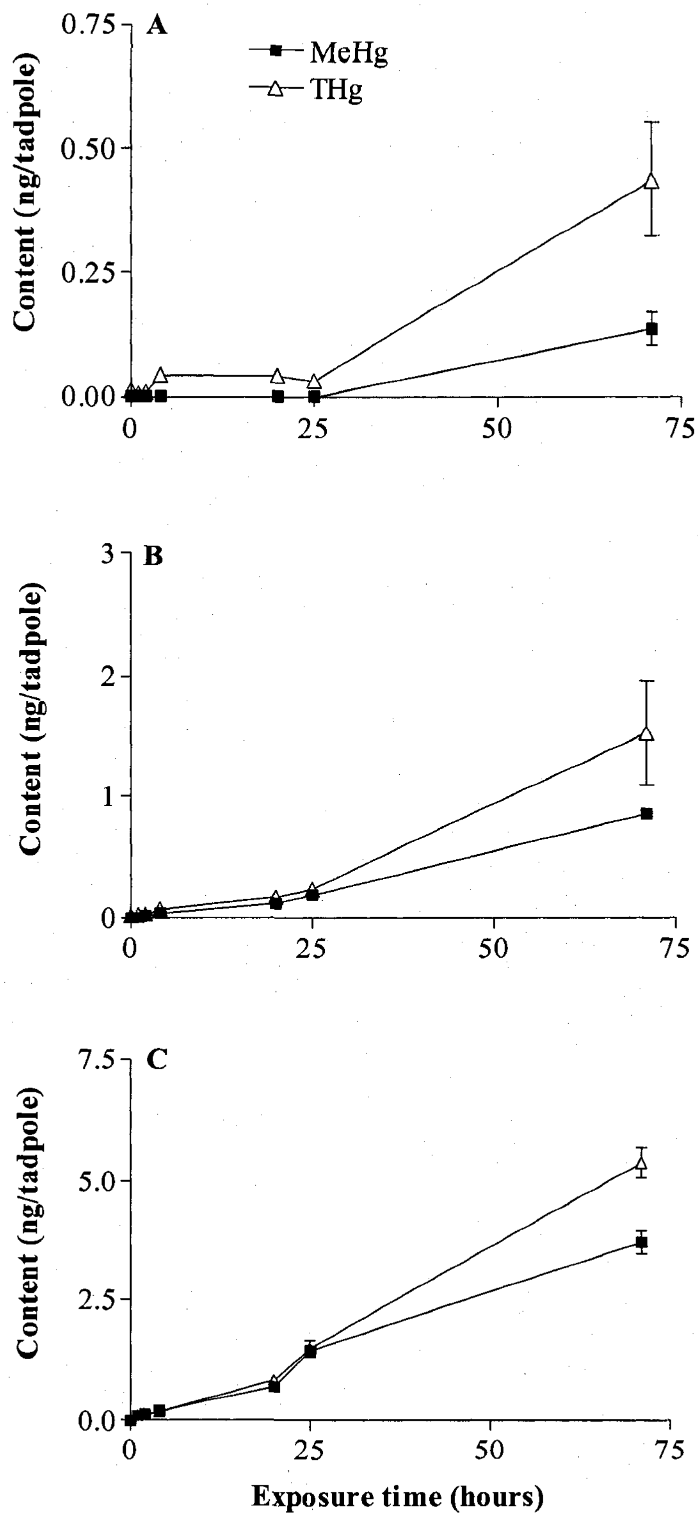


Figure 2.7. MeHg and THg content per tadpole over the first four days on the exposure experiment in the control (**A**), 50 ng/g (**B**) and 250 ng/g (**C**) treatment groups ($\pm$ SE). Time is expressed in hours with day 1= 0, 1, 2 and 4 hours; day 2= 20 and 25 hours; day 4=71 hours. Note different scales on the y axes. $n \geq 6$.

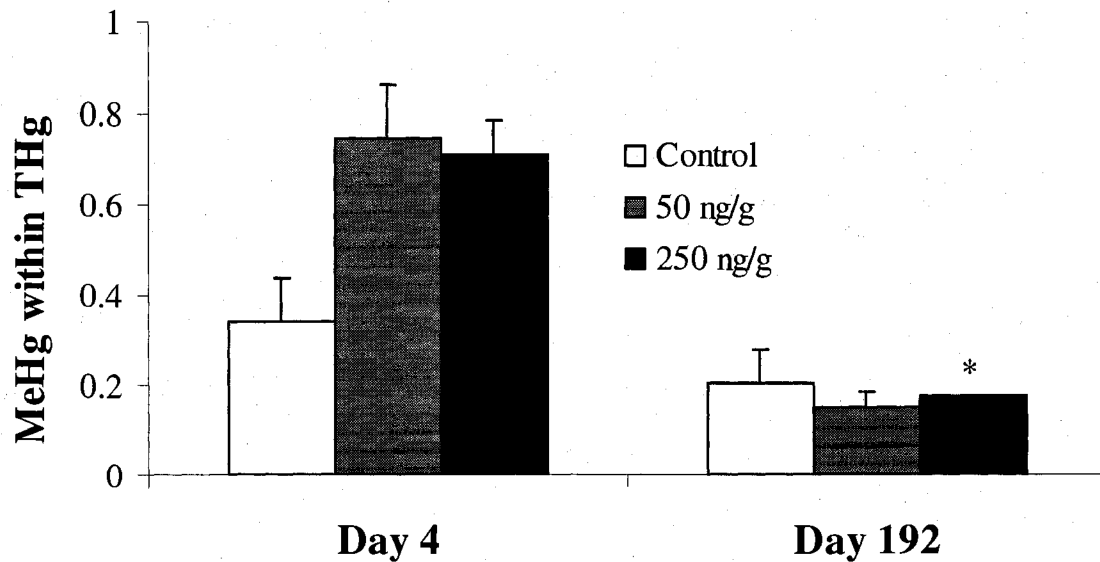


Figure 2.8. Proportion of MeHg within THg content for the control, 50 ng/g (low) and 250 ng/g (high) treatment groups (+ SE) during the initial accumulation phase (day 4) and on the final day of exposure (day 192). Mercury content was adjusted for body mass dilution. * Data for THg on the last day of exposure for the 250 ng/g treatment group reflects only one tail. n=8.

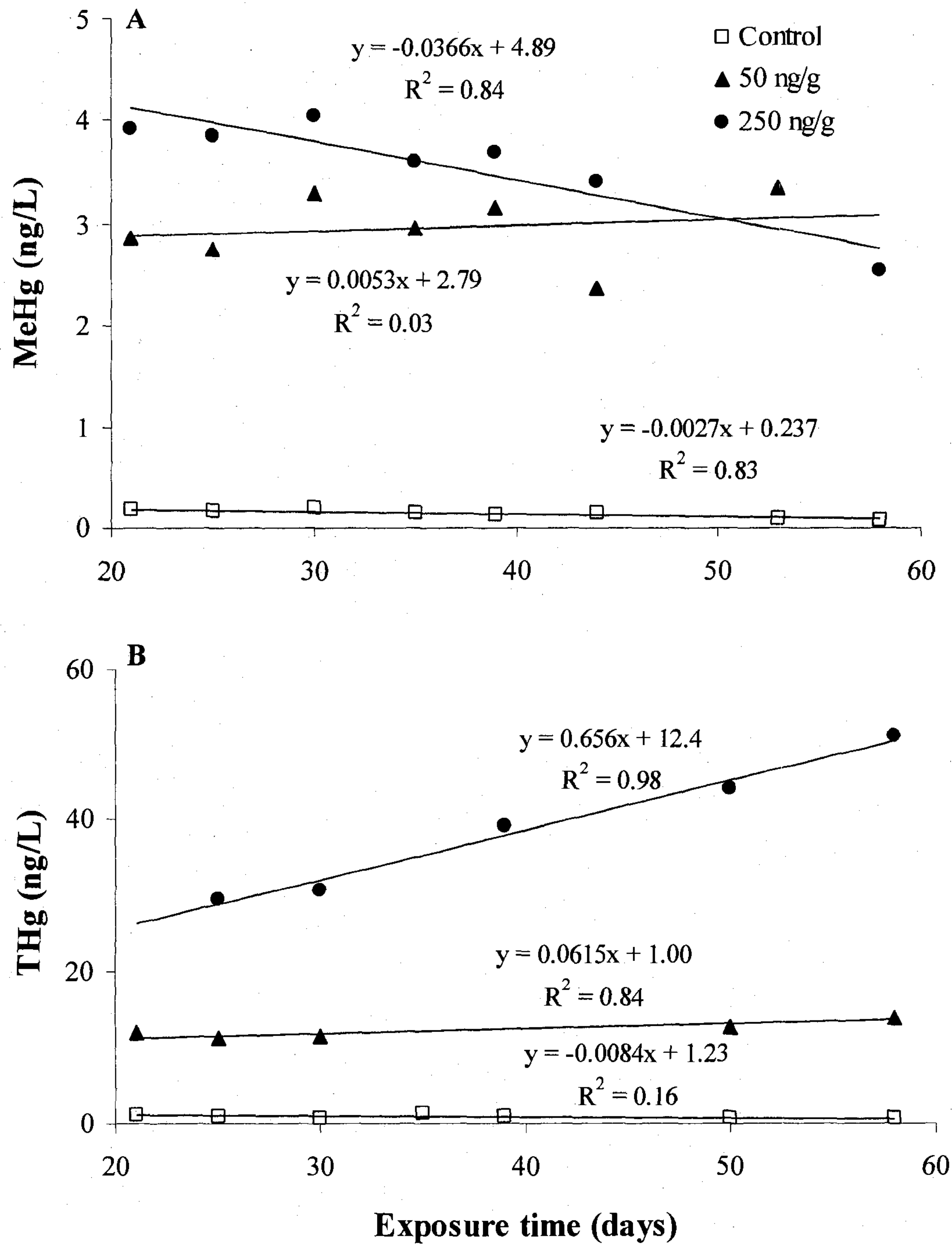


Figure 2.9. Average MeHg (**A**) and THg (**B**) concentrations in water obtained from control, 50 ng/g and 250 ng/g group tanks sampled between days 20 and 60 of exposure Water samples were taken 24 hours after the previous feeding to ensure that the water had been fully cleared of food by the tadpoles. Note different scales on the y axes. n=6.

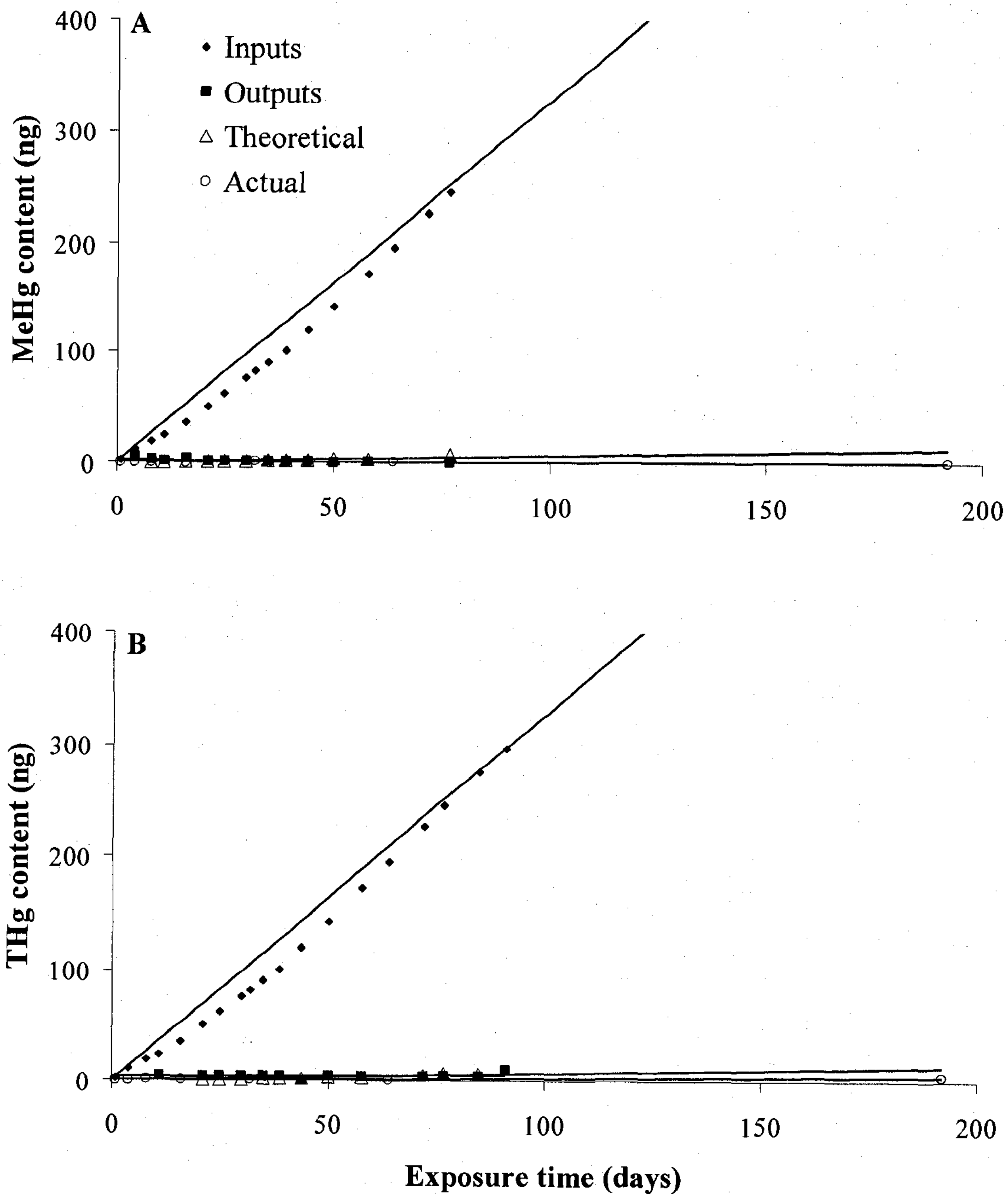


Figure 2.10. Mass balance for (**A**) MeHg content and (**B**) THg content in the control group. Inputs represent the amount of Hg in the food fed to tadpoles per day and outputs represent the amount of Hg removed through tank cleaning. Theoretical accumulation was calculated by subtracting outputs from inputs and dividing this value by the number of tadpoles per tank. Actual values represent measured values of Hg content per tadpole.

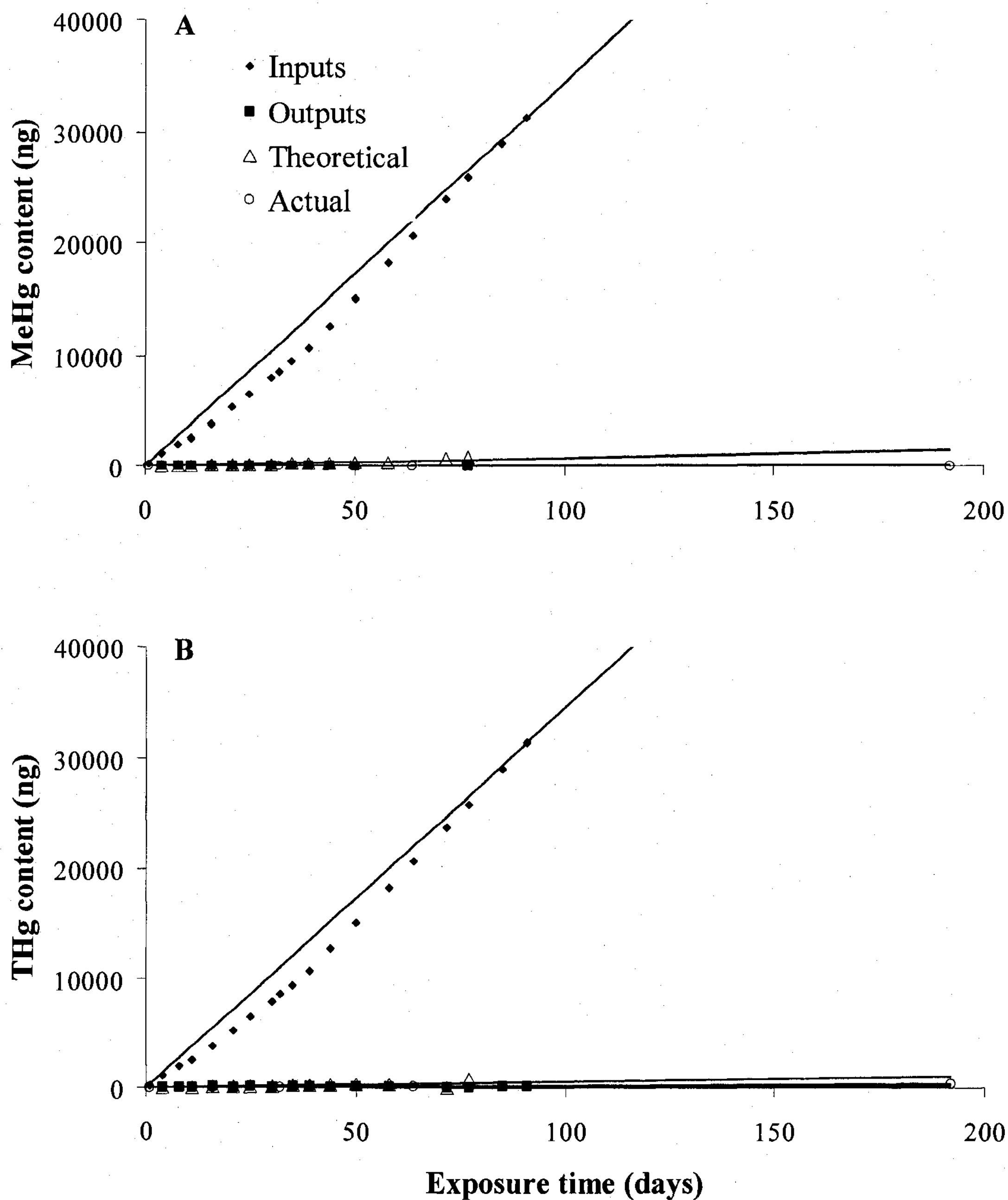


Figure 2.11. Mass balance for **(A)** MeHg content and **(B)** THg content in the 50 ng/g treatment group. Inputs represent the amount of Hg in the food fed to tadpoles per day and outputs represent the amount of Hg removed through tank cleaning. Theoretical accumulation was calculated by subtracting outputs from inputs and dividing this value by the number of tadpoles per tank. Actual values represent measured values of Hg content per tadpole.

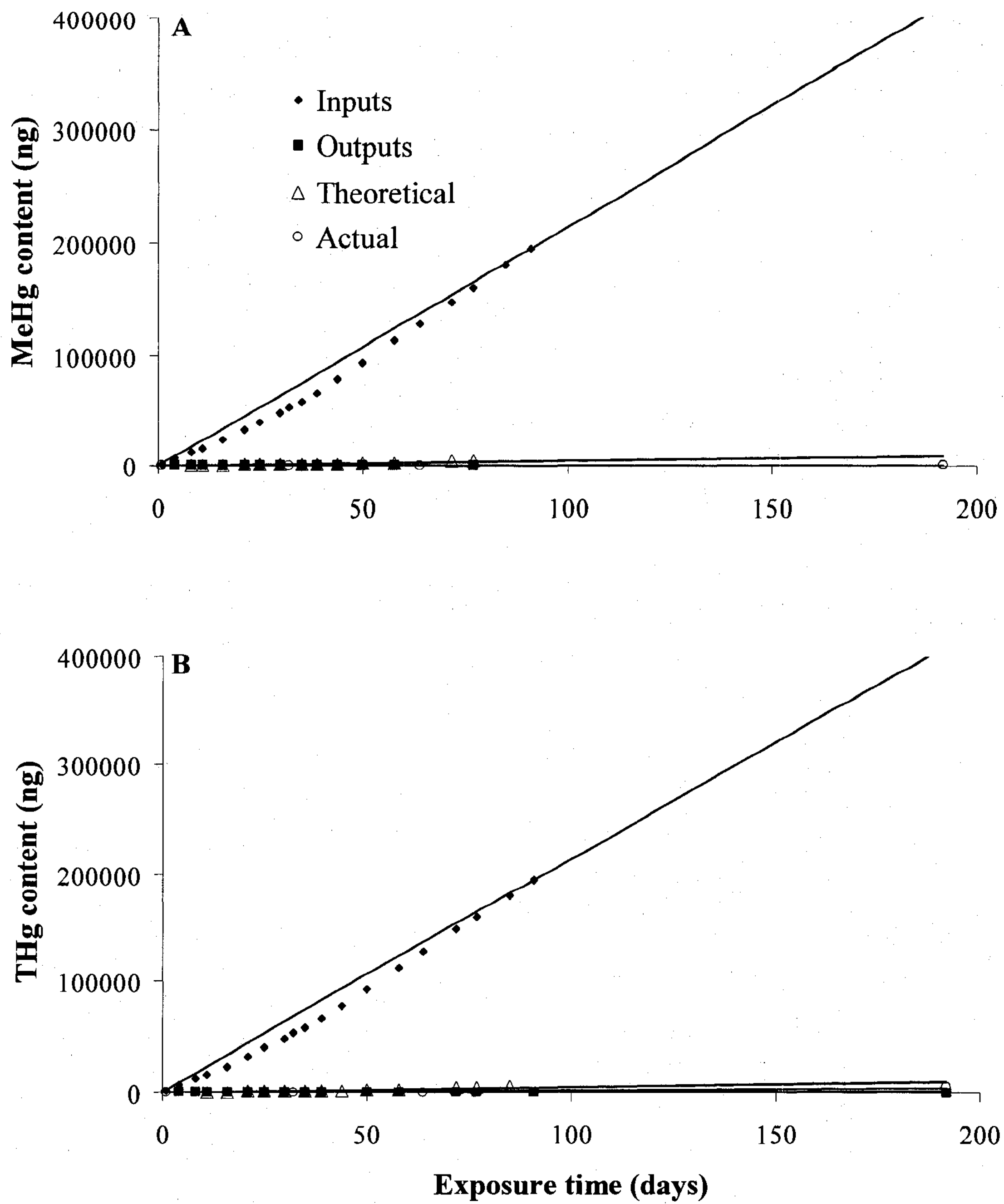


Figure 2.12. Mass balance for (**A**) MeHg content and (**B**) THg content in the 250 ng/g treatment group. Inputs represent the amount of Hg in the food fed to tadpoles per day and outputs represent the amount of Hg removed through tank cleaning. Theoretical accumulation was calculated by subtracting outputs from inputs and dividing this value by the number of tadpoles per tank. Actual values represent measured values of Hg content per tadpole.

CHAPTER 3

Developmental alterations and thyroid hormone levels in relation to level of dietary methylmercury exposure

3.1 Introduction

It has been well established that MeHg is toxic to many vertebrates as studies on its effects in humans and piscivorous wildlife have been carried out for many years. High level MeHg exposure has been shown to cause many adverse behavioural and physiological outcomes (Aulerich et al. 1974, Wren et al. 1987, Fjeld et al. 1998, Heinz and Hoffman 1998, Wolfe et al. 1998, Dansereau et al. 1999, Spalding et al. 2000a, Spalding et al. 2000b, Mela et al. 2007). The effects of acute exposure, however, may not be relevant to what organisms encounter in natural habitats. To date, it is unclear whether current environmental levels of MeHg pose health hazards to free-living wildlife (Scheuhammer 2007). Research on the effects of environmentally relevant levels of MeHg is warranted, especially in organisms such as amphibians that are directly exposed to MeHg within their habitats throughout sensitive periods of development (see Chapter 2). The present study aims to investigate the effects of chronic, low level MeHg ingestion on amphibian tadpoles and determine whether MeHg can act as an endocrine disruptor leading to changes in developmental rates and metamorphosis.

Endocrine disruption by environmental contaminants is currently a concern for the health of both humans and wildlife (Damstra et al., 2002). It is unknown, however, if MeHg exposure has the capacity to disrupt endocrine systems. Recent studies suggest that chronic, low level MeHg exposure could have effects not only on hormonal activity but also on cellular functions and gene transcription both *in vitro* and *in vivo*. For

example, *in vitro* exposure to low levels of MeHg has resulted in dissolution of microtubules and neuronal processes, apoptosis, and cell death in rat cerebellar granule neurons (Castoldi et al. 2000), decreases in ERα protein levels and alterations in growth-regulating signaling pathways in human breast cancer cells (Martin et al. 2003), and inhibition of D2 activity in mouse neuroblastoma and rat pituitary tumor cells (Mori et al. 2006a, Mori et al. 2007). *In vivo*, low level exposure has been shown to cause increases in muscarinic cholinergic receptor density and decreases in dopaminergic-2 receptor density in the brains of mink (Basu et al. 2005), decreased cholinesterase and monoamine oxidase activity in the brains of river otters (Basu et al. 2007), suppressed levels of plasma testosterone and 17ß estradiol in fathead minnows (Drevnick and Sanderheinrich 2003), and changes in deiodinase activity in the brains of fetal (Watanabe et al. 1999) and neonatal mice (Mori et al. 2006b). Any of the aforementioned changes due to MeHg exposure could have negative impacts on developing organisms, especially those that affect components of the hypothalamo-pituitary-thyroid axis which controls amphibian development and metamorphosis (Denver 1998b). Disruption within this axis has the potential to affect rates of development and thus future fitness (Crespi and Denver 2005).

In amphibians, rate of development is inversely related to larval growth rate and size at metamorphosis. Therefore, both a longer larval period and a smaller size at metamorphosis can impact fitness (Denver et al. 2002). Because in nature amphibian survival throughout development is generally low due to environmental factors such as predation, loss of habitat, and competition (Berven 1990), a longer time to metamorphosis may also compound these factors. A slower rate of metamorphosis may

also directly affect fitness by increasing the amount of time that tadpoles are in contact with, and therefore affected by, environmental contaminants such as MeHg.

The objective of this study was to determine whether exposure to environmentally relevant levels of MeHg could alter the normal progression of development in *Xenopus tropicalis*. Tadpoles were exposed to diets enriched with MeHg from the time of independent feeding (NF stage 45/46) until metamorphic climax (NF stage 60). Rate of metamorphosis, mortality, and size were recorded throughout the study. TH levels were also measured in order to assess potential disruption to the hypothlamo-pituitary-thyroid axis. We hypothesized that sublethal MeHg concentrations would disrupt the TH axis controlling metamorphosis leading to an increase or decrease in both growth rate and/or the time required for tadpoles to reach metamorphosis.

3.2 Methods

Details regarding laboratory rearing conditions, conditions of exposure, and experimental diets are described in Chapter 2 (2.2 Methods; *2.21 Breeding, 2.22 Conditions of exposure and 2.23 Experimental diets*).

3.21 Sampling

Animals were observed daily over the course of the exposure experiment. The number of deaths was recorded and dead animals were removed. Any physical abnormalities or behavioural changes were also noted. Lethargic behaviour was defined as a reduction in movement and decreased startle response. To avoid stressing the animals through physical manipulation (e.g., touching them), lethargic behaviour was

noted during normal interaction with the tadpoles (e.g., during feeding or tank cleaning etc.). When aquaria containing tadpoles are approached, a normal response (as seen in tadpoles from our breeding population) is to swim away from the side of the tank that is approached. Lack of response or general apathy towards an outside stimulus was recorded as lethargy. Resting and floating behaviours were defined as resting on the bottom of the tanks and floating on the surface of the water of the tanks, respectively. As *X. tropicalis* tadpoles are mid-column swimmers, both of these behaviours are considered to be abnormal. All abnormalities were noted descriptively and were considered to be significant when more that 50% of the population within a group was exhibiting these behaviours.

Developmental stages were also recorded to track the progression of tadpoles towards metamorphosis. Determination of developmental stages was based on the table describing the normal development of *Xenopus laevis* by Nieuwkoop and Faber (NF) (1994). These stages are based on various external and internal criteria. Tadpoles were sampled at NF stage 60, which marks the time immediately after limb morphogenesis and right before tail resorption, signaling the beginning of metamorphic climax. Age at metamorphosis was defined as the number of days required for tadpoles to reach metamorphic climax (NF stage 60). Tadpoles were euthanized using MS-222 (Sigma-Aldrich Canada Ltd., Oakville, ON) at a concentration of 1 g/L. For each tadpole, mass was measured using a Sartorius Corp. BP 121S balance (Edgewood, NY) and snout to vent length and tail length were measured with Lee Valley Tools Ltd. 88N62.54 digital calipers (Ottawa, ON). A subset of tadpoles per treatment group was snap frozen on dry ice and stored at -80°C for whole body TH measurement.

3.22 Thyroid hormone Extraction

TH was extracted from whole *X. tropicalis* tadpoles using methods adapted from Denver (1993) and references therein. Briefly, samples were homogenized in 3 volumes of methanol (MeOH) containing 1 mM propylthiouracil (PTU) to block endogenous deiodinase activity. Each sample was homogenized for 20 s using a Polytron homogenizer (Brinkmann Instruments Ltd., Mississauga, ON), then sonicated for 20 s using a Branson Sonifier (Branson Ultrasonics Co., Danbury, CT). An aliquot (200 µl) of the homogenate was removed and stored at -20°C for subsequent protein determination. $[I^{125}]$- T_3 (Perkin Elmer Inc., Waltham, MA) was used as a tracer for estimation of hormone extraction efficiencies. Free iodine was removed from the T_3 label prior to use with a Sep-Pak C_{18} cartridge (Waters Co., Milford, MA). The cartridge was activated with 1 ml MeOH and rinsed with 1 ml of 0.02 M phosphate-buffered saline (PBS; pH 7.4). The $[I^{125}]T_3$ was loaded onto the cartridge in a volume of 0.1 ml PBS, washed four times with 1ml PBS and eluted in a volume of 3 ml MeOH. The eluted tracer was diluted with MeOH-PTU to give 1000 cpm/200 µl. This volume was added to each sample homogenate and incubated for 15 min at room temperature. Samples were vortexed (VWR VX-2500, Mississauga, ON) for 12 min and centrifuged (IEC 3000R, Worcester, MA) for 20 min (1300 x g; 4°C). After centrifugation, the supernatant was decanted into tubes containing 5 ml chloroform (CH_3Cl) and extracted into an aqueous phase with 0.5 ml 2 N ammonium hydroxide (NH_4OH). The aqueous phase was removed and the extraction was repeated twice with the same CH_3Cl but new NH_4OH. The tissue pellet obtained above was kept in 1 ml of MeOH-PTU and was extracted three times as previously described. All aqueous phases were pooled and extracted a final time with an

equal volume of CH_3Cl. The final extract was loaded onto Bio-Rad Poly-Prep ion exchange chromatography columns (Mississauga, ON) which were prepared with Bio-Rad AG 1X2 chloride resin (200-400 mesh; 1.5 ml bed volume) in 0.2 M acetate buffer (pH 7) and equilibrated with 0.2 M acetate buffer (pH 7). The columns were then washed with the following series of solutions: 1) 2 ml acetate buffer (pH 7); 2) 2 ml of 1 mM ethanol (EtOH)-PTU; 3) 4 ml acetate buffer (pH 7); 4) 2 ml 1 mM EtOH-PTU ; 5) 2 ml acetate buffer (pH 7); 6) 2 ml acetate buffer (pH 4); 7) 2 ml acetate buffer (pH 3); 8) 2 ml 1% acetic acid; and 9) 2 ml 35% acetic acid. Samples were eluted from columns in 1.5 ml 70% acetic acid, dried in a Savant AE2010 Speedvac (Holbrook, NY), and resuspended in 200 μl of human steroid-free serum (MP Biomedicals Inc., Orangeburg, NY). Extraction efficiencies were based on the recovery of radiolabeled T_3 tracer. Samples were counted in a gamma counter (Packard Cobra™ auto-gamma®, Hewlett Packard Co., Mississauga, ON) and recoveries ranged from 30 to 70%.

3.23 Enzyme-Immuno Assay (EIA)

T_3 levels were measured by enzyme immunoassay (EIA) test kits for human serum (MP Biomedicals Inc., Orangeburg, NY) as per the manufacturer's instructions. Steroid-free serum was initially tested by EIA and no TH was detected (<0.2 ng T_3/ml). Serial dilutions of our sample extracts were also performed to ensure that parallelism with the standard curve supplied by the manufacturer was achieved. Because tadpoles were at metamorphic climax, the time when circulating levels of T_3 within the animals are highest, samples were diluted with steroid-free serum to ensure that T_3 levels fell within

the standard curve of the test kit. TH level measurements were corrected for dilution, volume, and recoveries.

3.24 Protein Determination

The 200 μL aliquot collected following tissue homogenization and sonification was used in the Bio-Rad Protein assay to measure protein levels. The microassay procedure for microtiter plates was performed as per the manufacturer's instructions. Due to the incompatibility of the methanol solvent in which the samples were homogenized with this protocol, samples were dried in a Savant AE2010 Speedvac and reconstituted in 800 μL ddH20. Samples were vortexed for approximately 60 s and centrifuged for 15 min (15000 x g; 4°C) and the supernatant was used in the assay. Bovine serum albumin (BSA) was serially diluted in ddH20 to provide the standard curve, and both the standard curve and samples were assayed in duplicate. The protein concentration measured was used to calculate the amount of protein per animal in mg.

3.25 Data analysis

All data were assessed for normality (Lillefore's test) and homogeneity of variance (Levene's test) and were transformed if necessary to meet parametric assumptions. Percentage of mortality and metamorphosis (adjusted for mortality) were tested using log linear models as both data sets followed a Poisson distribution. Categorical variables used for analysis were exposure group (control, low treatment, high treatment) and outcome (alive vs. dead; metamorphosis vs. no metamorphosis). Age, the number of days between metamorphs, tail length, snout to vent length, mass, and TH

levels were analyzed using one-way ANOVA followed by Tukey's pair-wise comparisons. Significance was set at $p \leq 0.05$. Analyses were performed using Systat 10 software (SPSS Inc., Chicago, IL).

3.3 Results

Experimentation was terminated after 192 days, when 94% of the high MeHg treatment tadpoles had been removed from the study through death or metamorphosis. Remaining tadpoles were used for body burden analysis (see Chapter 2) or measurement of TH levels.

3.31 Mortality

Incidence of mortality in all three groups was relatively low until approximately day 35 of exposure when it began to increase (Figure 3.1). Exposure day 35 corresponds to 40 days post fertilization for the tadpoles. We have noted on three previous occasions (in a preliminary MeHg exposure experiment as well as two experiments conducted by other members of the laboratory) that an increase in mortality for *X. tropicalis* occurs at this time, indicating that this is a sensitive time of development. At this time, the majority of tadpoles in all groups were at NF stages 50/51 which is a period of cell proliferation and rapid growth. Mortality remained similar in all three groups until approximately day 70 of exposure when it increased in the high MeHg group and continued to increase steadily until the final day of exposure. Mortality remained similar for the control and low MeHg groups throughout the exposure. On the final day of exposure, the percent

mortality in the high MeHg group was significantly higher than the percent mortality in the control group (χ^2=326.1; df=1; p<0.0001).

3.32 Metamorphosis

Percentage of tadpoles to reach metamorphosis was significantly greater in the high MeHg group when compared to the control group (χ^2=41.95; df=1; p<0.0001; Figure 3.2), but was not significantly different between the low MeHg group and control group. Overall percentages of metamorphosis were corrected for mortality by dividing the number of metamorphs by the number of surviving tadpoles. Although the percentage of metamorphosis in the high MeHg group was elevated, this a consequence of the high mortality experienced by this group. Few tadpoles survived long enough to reach metamorphosis. Those that did survive, however, were more likely to reach metamorphic climax.

3.33 Age

Age at metamorphosis was not significantly different between groups (F=0.73; df=2; p=0.484; R^2=0.01; Figure 3.3). The number of days between each tadpole reaching climax, however, was significantly longer in the high MeHg group (F=6.68; df=2; p=0.001; R^2=0.08; Figure 3.4).

3.34 Size

Tadpole mass was significantly greater in the high MeHg group when compared to the control group (F= 13.86; df=2; p<0.0001; R^2=0.16; Figure 3.5). Mean snout to vent

length (Figure 3.6) and tail length (Figure 3.7) were also greater for tadpoles in the high treatment group when compared to those in the control group ($F= 5.57$; $df=2$; $p=0.004$; $R^2=0.07$ and $F= 26.29$; $df=2$; $p<0.0001$; $R^2=0.26$, respectively). Differences in weight, snout to vent length, and tail length were not significantly different between the low MeHg and control groups.

3.35 Behavioural and morphological changes

Several behavioural and morphological abnormalities were noted through qualitative observation. Tadpoles in both the low and high MeHg groups exhibited lethargy beginning early in the exposure experiment (Table 3.1). Low MeHg group tadpoles first exhibited lethargy (>50% of the population) beginning at day 23 of exposure. This behaviour was absent after day 51 of exposure. Tadpoles in the high MeHg group exhibited lethargy slightly later (day 35), however, once it was observed it continued until the final day of exposure (day 192). The onset of this behavioural abnormality corresponds to a sensitive period of development for *X. tropicalis* tadpoles (see *3.31 Mortality*). Therefore, the presence of MeHg may have contributed to its appearance. A significant number of tadpoles in the high MeHg group were also observed to be resting on the bottom of the tanks or floating on the surface of the water (Table 3.1). Although the onset of the resting behaviour occurred earlier than the floating behaviour (day 34 and day 70, respectively) both were observed in greater than 50% of the population for the remainder of the experiment. The appearance of the resting behaviour also occurred during the aforementioned window of developmental sensitivity whereas the floating behaviour appeared at the time point when mortality in the high MeHg group

began to deviate significantly from the control or low MeHg groups (day 70; see Figure 3.1). Tadpoles in the low MeHg group were not observed to be resting on the bottom of the tanks or floating on the surface of the water and tadpoles in the control group did not exhibit any behavioural abnormalities.

A number of tadpoles had increased skin pigmentation (Figure 3.8) and the frequency of this occurrence increased with increasing treatment level. Dissection of NF stage 60 tadpoles revealed that several tadpoles also exhibited hepatic abnormalities. The livers of these tadpoles were greatly enlarged and highly pigmented when compared to the livers of animals from the control group. Again, the frequency of this occurrence increased with increasing treatment level with tadpoles in the high MeHg group having the highest proportion of abnormalities.

3.36 Thyroid hormone levels

T_3 levels were measured in whole body tadpoles at metamorphosis. Mean T_3 levels adjusted for volume, recovery of the radioactive tracer, body mass, and protein levels indicated that there were no significant differences in levels between groups (Figure 3.9; Body mass: $F=0.41$; $df=2$; $p=0.671$; $R^2=0.03$; Protein: $F=0.16$; $df=2$; $p=0.853$; $R^2=0.01$).

3.4 Discussion

Chronic exposure of tadpoles to higher environmentally relevant levels of dietary MeHg negatively affected tadpole survival and development. We observed that rates of mortality were similar between all groups until approximately day 70 of exposure when

mortality increased significantly in the high treatment group. On this day, development was variable between all groups with tadpoles ranging from NF stages 52 to 60. This day of exposure corresponds to the period of time when MeHg levels in tadpoles in the control and low MeHg groups reached a steady state and levels in the high MeHg group continued to rise over time (see Chapter 2). This result suggests that the tadpoles in the high MeHg group have reached a threshold at this time, beyond which they are unable to further detoxify MeHg, leading to an increased mortality.

On the final day of exposure, cumulative percent mortality in the high MeHg group was 94% compared to 45% and 47% (respectively) in the control and low MeHg groups. The percentage of mortality for the control group was not unexpected given the length of the exposure experiment. Mortality of tadpoles in a laboratory setting can be highly variable, for example ranging from 18% in one experiment with *X. tropicalis* (Fort et al. 2004) to 79% in a different experiment with *Rana clamitans* (Green frog; Coady et al. 2004). In a similar chronic exposure experiment conducted by Gibson (2005), *X. tropicalis* tadpoles in the control group experienced approximately 50% mortality after 77 days of development, whereas tadpoles exposed through diet to 50 ng/g and 500 ng/g wet wt. of MeHg experienced approximately 35% and 72% mortality, respectively. These differences in mortality are most likely due to differences in experimental design and should be taken into account when interpreting results. For example, Gibson (2005) changed water daily and it was not buffered with a Ringer's solution. *X. tropicalis* tadpoles are very sensitive to changes in water temperature and salinity. Therefore, less frequent water changes (removing no more than 50%) using buffered water greatly reduces mortality (Hirsch et al. 2002). By performing less frequent water changes using

water buffered with a 1x20 Ringer's solution, we have also observed a tadpole mortality rate similar to that in our control group when using the same breeding pairs of *X. tropicalis* in other experiments in the laboratory (personal observation).

The percentage of metamorphosis in the high MeHg group was significantly greater than that of the control group when adjusted for mortality. Although it appears that a large percentage of high MeHg group tadpoles reached metamorphosis, this is a result of the high mortality experienced by this group. I speculate that the few tadpoles that were able to survive high MeHg exposure were also resilient enough to reach metamorphic climax. Although age at metamorphosis was not significantly different between groups, both the span of time between each tadpole reaching metamorphosis and mass at metamorphosis were greater in the high MeHg group when compared to the control group. On average, tadpoles in the control and low MeHg groups reached metamorphic climax every two days whereas tadpoles in the high MeHg group reached metamorphosis every five days. Although this could be interpreted as a consequence of high mortality in the high MeHg group, it could also be indicative of developmental delays prior to metamorphosis. This is also supported by the observation that tadpoles in the high MeHg group were 27% larger than those in the control group, which is inconsistent with what is observed in tadpoles undergoing accelerated metamorphosis. Tadpole development tends to be inversely related to growth rate. Therefore, tadpoles experiencing developmental delays as a result of numerous environmental challenges have been found to be larger at metamorphosis (Crespi and Denver 2005). Our results suggest that tadpoles in the high MeHg group are delayed during some stage(s) of

development and as a result have more time to grow and are larger at metamorphosis than tadpoles exposed to low levels of MeHg.

During the normal developmental progression towards metamorphosis, *X. tropicalis* tadpoles normally take 6 days to reach metamorphic climax (NF stage 60) once forelimb emergence (NF stage 58) has been initiated (Rowe et al. 2002). In our experiment we observed that it would take high MeHg group tadpoles as long as 21 days to reach climax once forelimb emergence had begun. These observations are consistent with the results of several other experiments examining the timing of developmental arrest in tadpoles exposed to endocrine disrupting chemicals. For example, Iwamuro et al. (2003) found that *Xenopus laevis* tadpoles exposed to a combination of 10^{-5} M bisphenol A (BPA) and 10^{-7} M T_4 (the precursor to T_3) in water for 21 days beginning at NF stage 52, were developmentally delayed at NF stages 58 and 59. Balch et al. (2006) found that *X. laevis* tadpoles exposed to 5000 μg/g of a commercial pentabromodiphenyl ether (PDE) mixture through the diet beginning at NF stage 51 also experienced arrested development at NF stage 58 and had altered skin pigmentation (increased pigmentation was observed in a high proportion of high treatment tadpoles in our study). At a lower level of 1000 μg/g of PDE, metamorphosis was delayed at NF stage 58, although tadpoles were able to recover and reach metamorphic climax. The tadpoles in our high MeHg group that exhibited a delay in development between stages 58 and 60 and survived treatment, were also able to recover and metamorphose.

Our measurements of T_3 levels in the low and high MeHg groups at metamorphic climax indicated that they did not differ from those in the control group. This suggests that tadpoles that survived high MeHg exposure could recover from a developmental

delay. The THs T_4 and T_3 are the primary morphogens controlling amphibian metamorphosis. All changes occurring during larval development are induced by T_4 which is enzymatically converted by deiodinase enzymes in target tissues to T_3, the most biologically active form of TH (Denver et al. 2002). The T_3 binds to thyroid receptors that function as ligand-inducible transcription factors and initiates tissue-specific development (Tata 1999). Changes in or inhibition of any of the aforementioned components of the thyroid axis have the capacity to delay or arrest development and several mechanisms have been proposed. For example, it is thought that the metabolites of PDE's bind to transport proteins and reduce the amount of T_4 transported to target cells (Meerts et al. 2000) whereas developmental delays as a result of exposure to BPA have been demonstrated to result from the suppression of TR gene expression (Iwamuro et al. 2003). In mammals, such as neonatal mice, exposure to MeHg throughout development has been shown to decrease D3 activity, which can lead to decreased inactivation of both T_4 and T_3 (Mori et al. 2006b). To date the mechanisms responsible for disruptions to the TH axis by MeHg remain to be elucidated. However, it is clear from the wide degree of variability in mechanisms responsible for the disruption of this axis by other chemical compounds that no one mechanism holds true in all circumstances and that more research is required.

Several behavioural and physiological abnormalities were observed in this study. Lethargy, resting on the bottom of the tanks, and floating on the surface of the water of the tanks were observed in a high proportion of the high MeHg group tadpoles. These behaviours are most likely attributable to the toxic effects of MeHg within the brain (Aulerich et al. 1974, Wren et al. 1987). In the developing brain, MeHg has the capacity

to disrupt normal pathways of development such as the way in which neurons move from the site of growth to the final site of function, the formation of interneuronal contacts, and the apoptotic death of neurons (Anderson and Swanson 2000). As all of these events are necessary for normal development in all organisms, MeHg may irreparably harm the central nervous system, leading to behavioural and functional changes. Although lethargic behaviour was also observed in the low treatment group early on in the experiment, in the past we have seen that incidences of lethargic behaviour and mortality increase at approximately day 40 of age in unexposed tadpoles from our normal breeding population. In the current study, tadpoles in all groups were at NF stages 50/51 at this time, which is a period of cell proliferation and rapid growth (Thors et al. 1982) and may be a particularly sensitive period of development. Therefore, it is possible that tadpoles were lethargic during these stages to conserve enough energy for both basic physiological processes and detoxification.

Increased skin pigmentation and enlarged, pigmented livers were observed in a high proportion of tadpoles from the high MeHg group and to a much lesser extent in tadpoles from the low MeHg group. I theorize that both abnormalities are potential markers of MeHg toxicity. Skin pigments such as melanins may play a defensive role in metal detoxification in amphibians. The mechanisms by which this occurs, however, are not fully understood (Sparling et al. 2000). Although little is known about hepatic abnormalities as a result of MeHg exposure in amphibians, several studies have observed such abnormalities in fish. For example, *Hoplias malabaricus* (Tiger fish) fed MeHg contaminated prey fish exhibited leukocyte infiltration, increased pigmentation, necrotic areas and lesions in the liver as well as cytoskeleton disorganization (Mela et al. 2007).

These results suggest a strong toxic effect in liver cells. *Esox lucius* (Northern pike) sampled from Isle Royale, Michigan with concentrations of THg in skin-on fillets ranging from 0.069 to 0.622 μg/g (wet wt.), presented variations in liver pigmentation that were positively related to liver THg concentration (Drevnick et al. 2008). The pigment causing variation in liver color was identified as lipofuscin, which results from lipid peroxidation of membranous organelles and has been also been suggested as a potential biomarker for Hg toxicity.

Very few studies have examined the effects of environmentally relevant levels of dietary MeHg on tadpole development, and few have been conducted over such an extensive period of time as in this laboratory study. Of those that have been conducted, low level exposure to MeHg (Unrine et al. 2004, Gibson 2005: 50 ng/g dry/wet wt.) has been shown to increase rates of metamorphosis, whereas high level exposure (Gibson 2005: 500 ng/g wet wt.) has been shown to have negative impacts on developmental rate. Amphibians, like most organisms, have the ability to modify their behaviour, morphology, and physiology in response to changing environments through phenotypic plasticity (Denver 1999). We hypothesized that sublethal MeHg concentrations would disrupt the TH axis controlling metamorphosis leading to an increase or decrease in both growth rate and/or the time required for tadpoles to reach metamorphosis, as has been observed in previous studies. For example, Unrine et al. (2004), found that *Rana sphenocephala* tadpoles ingesting a diet of 3300 ng/g dry wt. inorganic Hg, with a measured level of 50 ng/g dry wt. of MeHg, throughout development, had an increased rate of metamorphosis (as measured by a decrease in days to hindlimb and forelimb emergence). Similarly, Gibson (2005) found that *X. tropicalis* metamorphosis increased

significantly with exposure to 50 ng/g MeHg wet wt. alone. We did not, however, observe differences in metamorphic rate in the low treatment group of our study. Accelerated metamorphosis in tadpoles in the Gibson (2005) study may have resulted from differences in experimental design, such as environmental and handling stress on the tadpoles, and not MeHg exposure. Similarly, our results may differ from those of Unrine et al. (2004) because of differences in species sensitivity to MeHg or because of the high level of inorganic Hg used in their study.

In summary, tadpoles in the high MeHg group experienced high levels of mortality and abnormalities, suggesting that levels of MeHg representative of those found in aquatic environments have the capacity to overwhelm detoxification mechanisms. They also presented an increased span of time between each tadpole reaching metamorphosis and an increased body mass when compared to the control group, which suggests a delay at some stage in development. This delay most likely occurred before climax because surviving tadpoles were able to metamorphose, and levels of T_3 at climax were not significantly different from those in the control group. This is further supported by the observation of a development delay in the stages between forelimb emergence and the initiation of metamorphosis. We did not detect any effects of MeHg on T_3 levels in this study. While measuring whole body levels of T_3 can be indicative of major upsets in the thyroid system, is it not a sensitive measure, as it does not take into account variations in locally produced T_3, which is what drives tissue differentiation in amphibians (Buchholz and Hayes 2005). Future studies should therefore focus on determining at which level of the thyroid axis MeHg induced disruption of development occurs. This

could include measurements of deiodinases and TRs using gene expression or protein analysis at critical stages of development.

Table 3.1. Behavioural abnormalities observed in *X. tropicalis* tadpoles chronically exposed to MeHg. Initial and final days of exposure when tadpoles in each group exhibited the behaviour are indicated.

	Control		50 ng/g		250 ng/g	
Day	**Initial**[a]	**Final**[b]	**Initial**	**Final**	**Initial**	**Final**
Lethargy	NA[c]	NA	23	51	35	192
Resting	NA	NA	NA	NA	34	192
Floating	NA	NA	NA	NA	70	192

[a] where >50% of tadpoles exhibited the behaviour
[b] where >50% of tadpoles exhibited the behaviour
[c] NA= not apparent

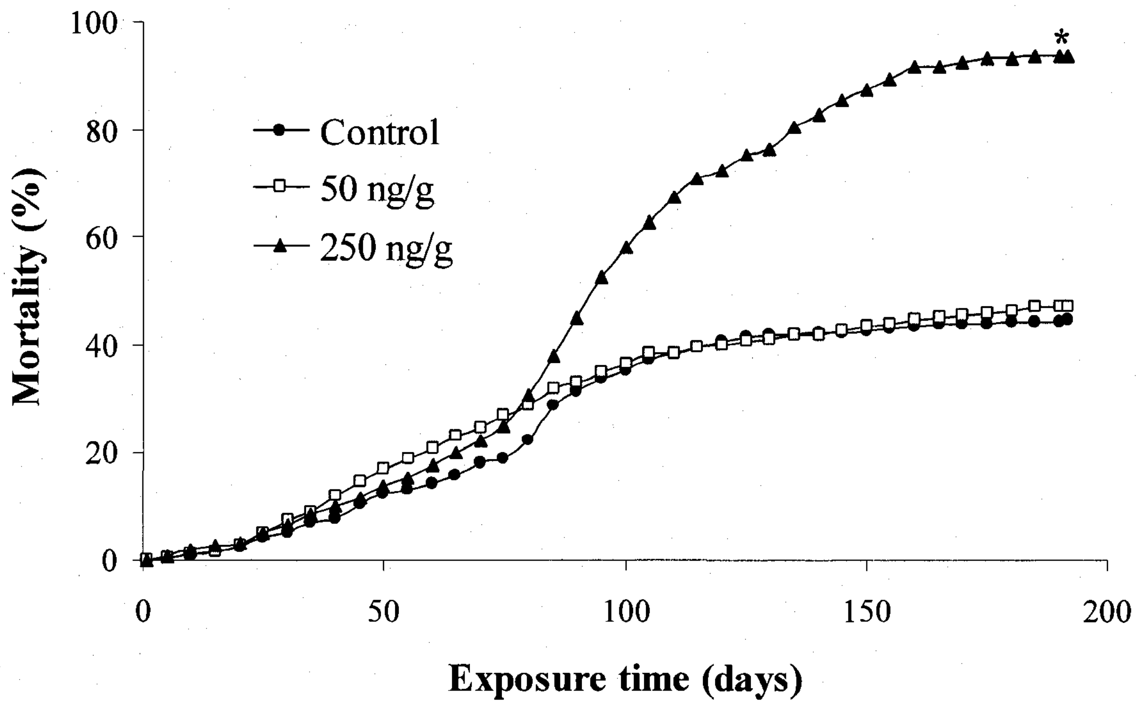


Figure 3.1. Percentage of mortality over time for *X. tropicalis* tadpoles chronically exposed to MeHg. Data were assessed by log linear model (significance at $p \leq 0.05$). Asterisk (*) denotes a significant effect of the 250 ng/g treatment ($p<0.0001$) on mortality on day 192. n=227, 233 and 501 for the control, 50 ng/g and 250 ng/g groups, respectively.

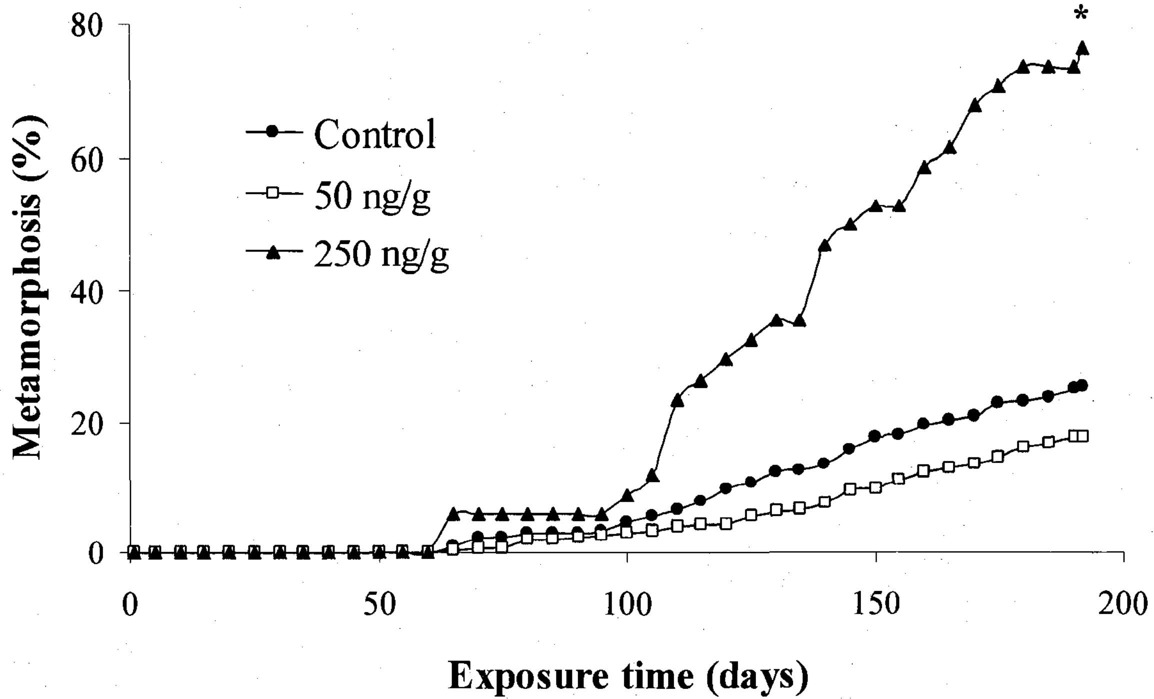


Figure 3.2. Percentage of metamorphosis adjusted for mortality over time for *X. tropicalis* tadpoles chronically exposed to MeHg. Data were assessed by log linear model (significance at $p \leq 0.05$). Asterisk (*) denotes a significant effect of the 250 ng/g treatment ($p<0.0001$) on mortality on day 192. n=71, 54 and 26 for the control, 50 ng/g and 250 ng/g groups, respectively.

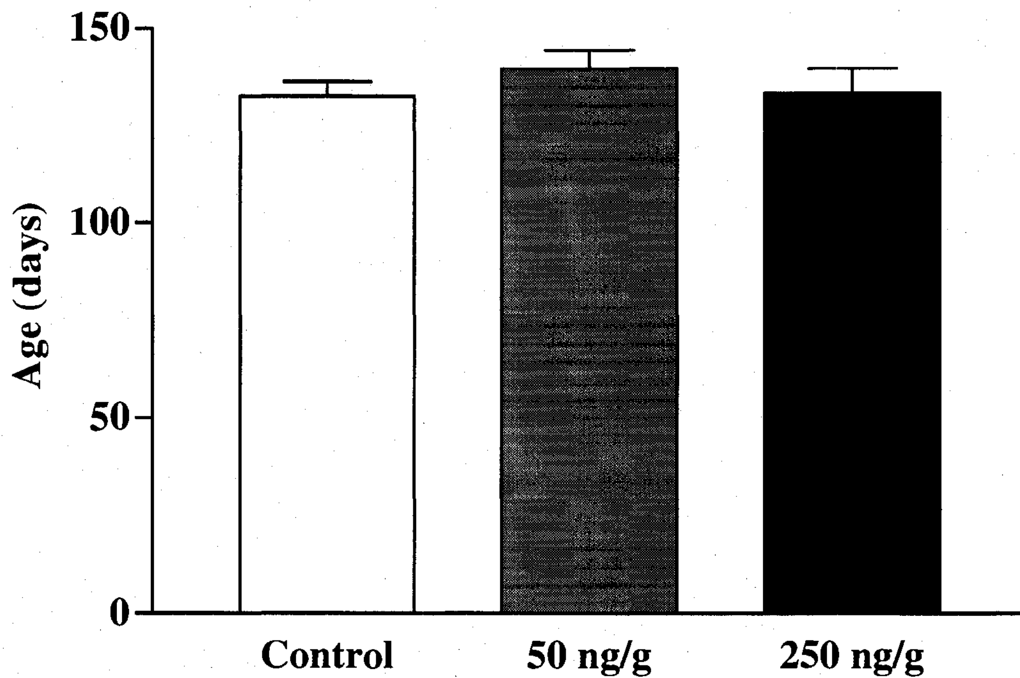


Figure 3.3. Age at metamorphosis for *X. tropicalis* tadpoles chronically exposed to MeHg. Data were assessed by one-way ANOVA (significance at $p \leq 0.05$).Values represent mean +SEM (n=71, 54 and 26 for the control, 50 ng/g and 250 ng/g groups, respectively). Ages were not significantly different between groups.

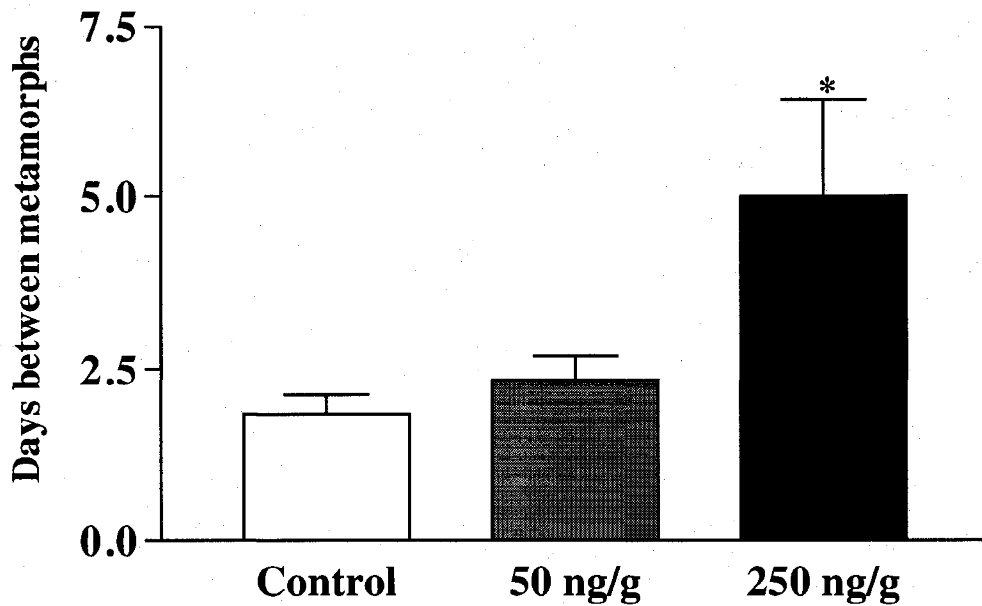


Figure 3.4. Number of days between each tadpole reaching metamorphic climax for *X. tropicalis* tadpoles chronically exposed to MeHg. Data were ln transformed to meet assumptions of normality and homogeneity and were assessed by one-way ANOVA followed by Tukey's pair-wise comparisons (significance at $p \leq 0.05$). Values represent mean +SEM (n=71, 54 and 26 for the control, 50 ng/g and 250 ng/g groups, respectively). Asterisk (*) denotes a significant difference compared to control (p=0.001).

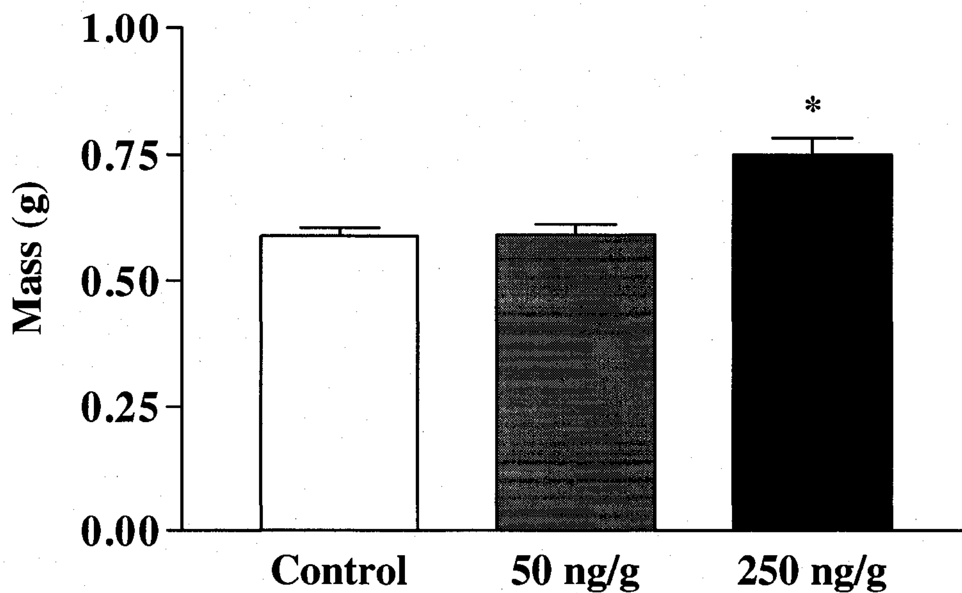


Figure 3.5. Body mass at metamorphic climax for *X. tropicalis* tadpoles chronically exposed to MeHg. Data were assessed by one-way ANOVA followed by Tukey's pair-wise comparisons (significance at $p \leq 0.05$). Values represent mean +SEM (n=71, 54 and 26 for the control, 50 ng/g and 250 ng/g groups, respectively). Asterisk (*) denotes a significant difference compared to control ($p<0.0001$).

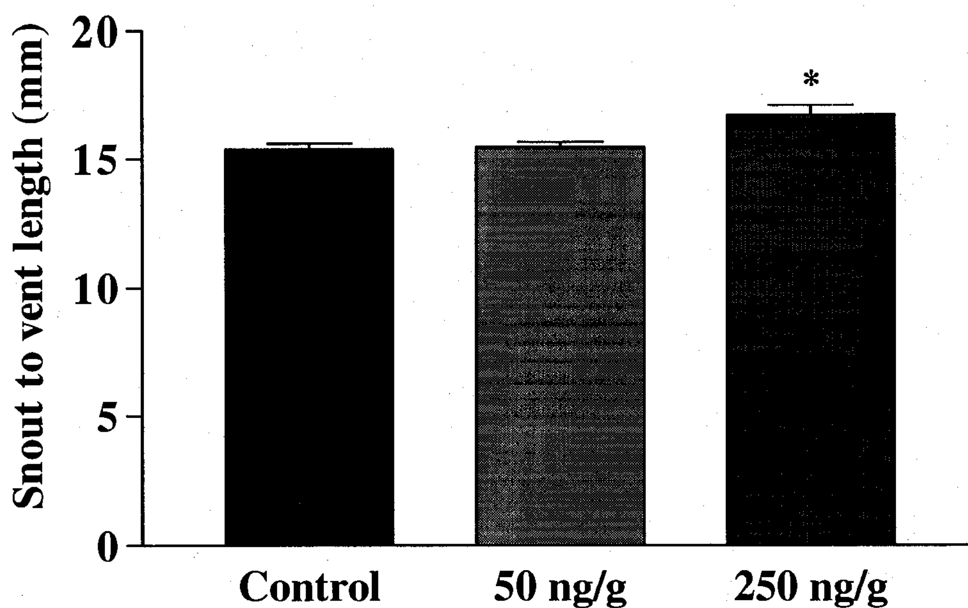


Figure 3.6. Snout to vent length at metamorphic climax for *X. tropicalis* tadpoles chronically exposed to MeHg. Data were ln transformed to meet assumptions of normality and homogeneity and were assessed by one-way ANOVA followed by Tukey's pair-wise comparisons (significance at $p \leq 0.05$). Values represent mean +SEM (n=71, 54 and 26 for the control, 50 ng/g and 250 ng/g groups, respectively). Asterisk (*) denotes a significant difference compared to control (p=0.004).

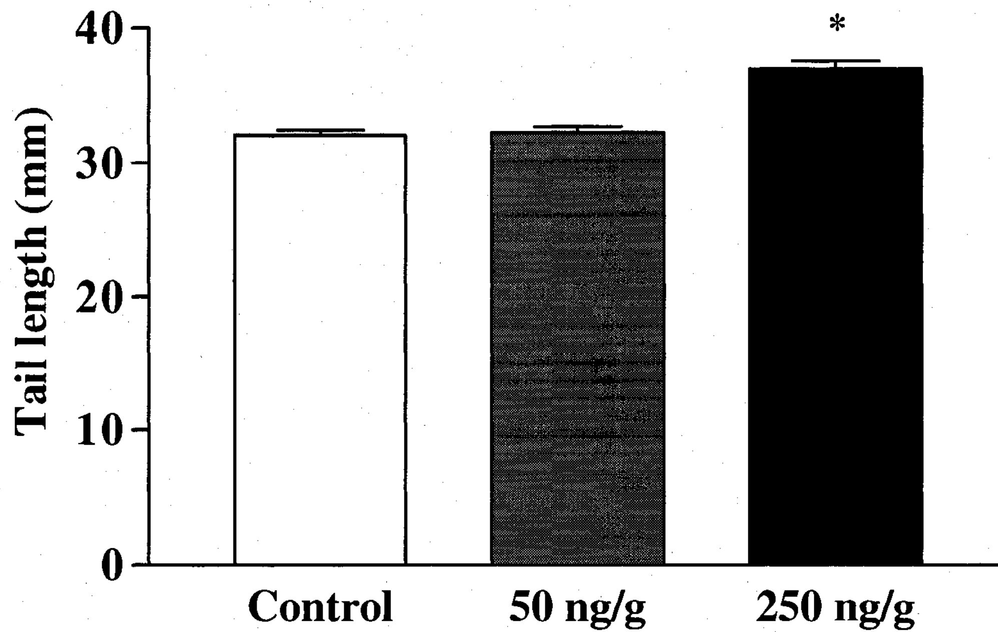


Figure 3.7. Tail length at metamorphic climax for *X. tropicalis* tadpoles chronically exposed to MeHg. Data were assessed by one-way ANOVA followed by Tukey's pair-wise comparisons (significance at $p \leq 0.05$).Values represent mean +SEM (n=71, 54 and 26 for the control, 50 ng/g and 250 ng/g groups, respectively). Asterisk (*) denotes a significant difference compared to control ($p<0.0001$).

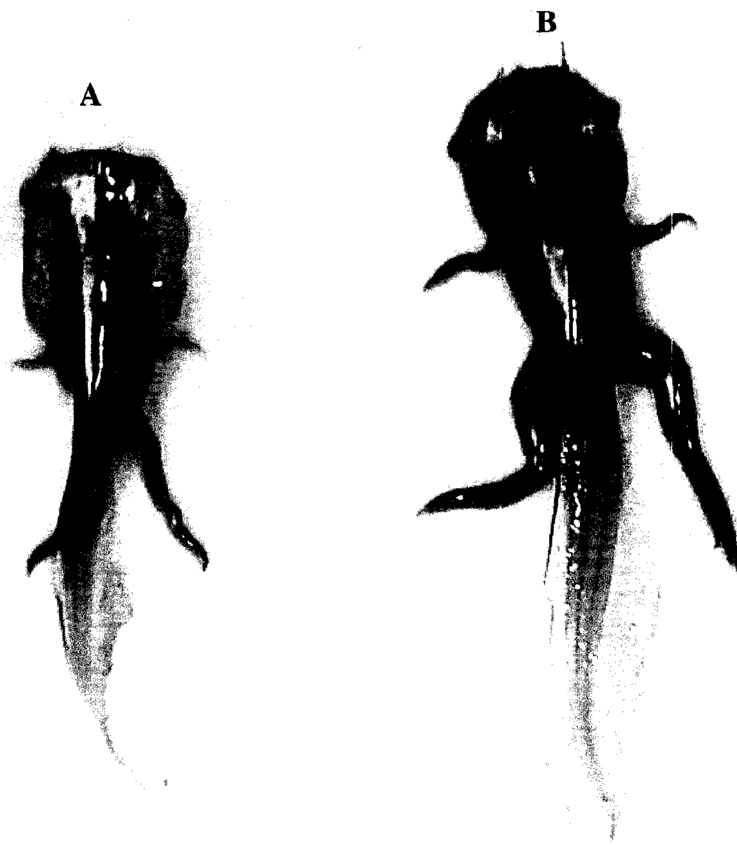


Figure 3.8. Differences in pigmentation between tadpoles at metamorphic climax (NF stage 60) from **A**) the control group, and **B**) the 250 ng/g treatment group. Note the difference in size between the tadpoles.

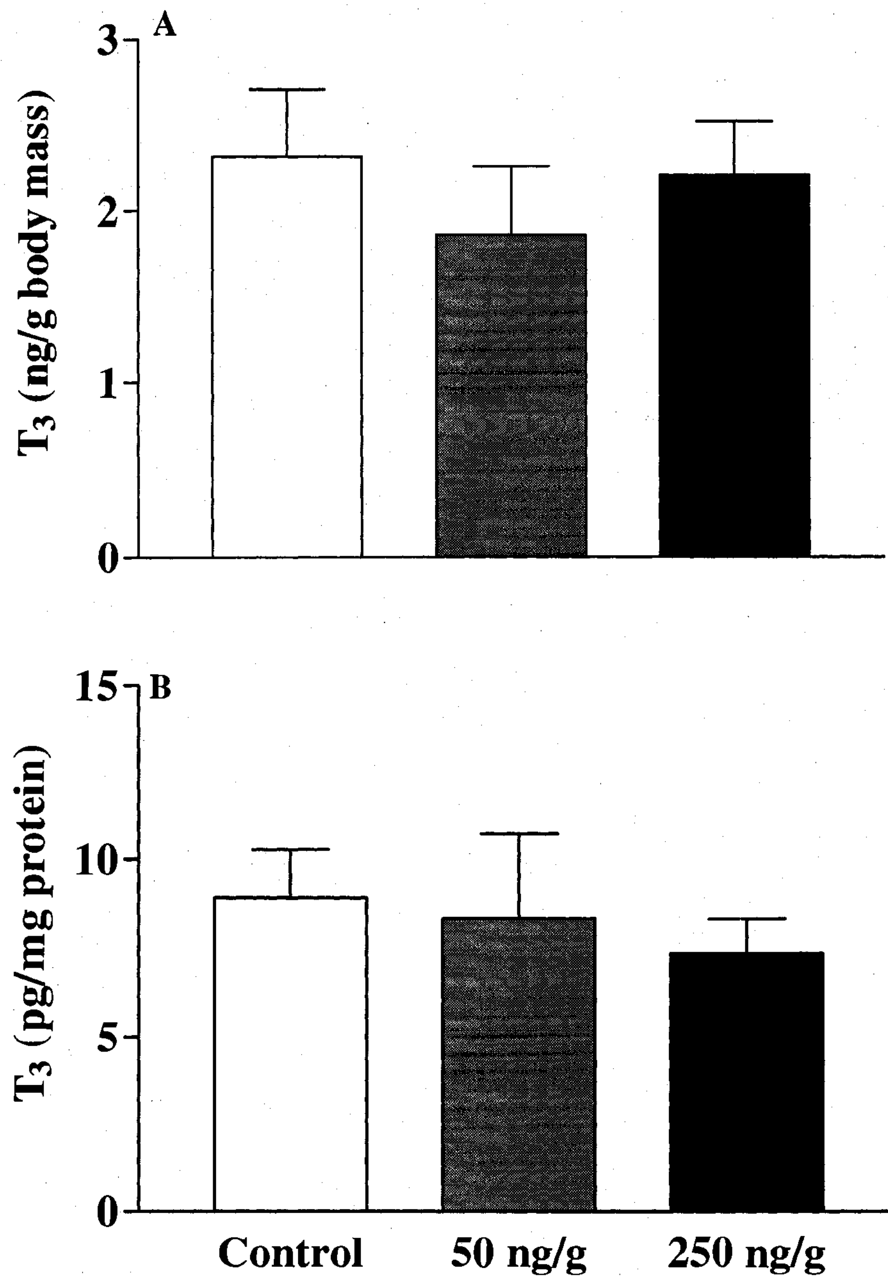


Figure 3.9. Whole body T_3 levels adjusted for (**A**) body mass at metamorphosis and (**B**) protein content in *X. tropicalis* tadpoles chronically exposed to MeHg. Data were assessed by one-way ANOVA (significance at $p \leq 0.05$). Values represent mean +SEM (n=9, 11 and 8 for the control, 50 ng/g and 250 ng/g groups, respectively). Levels of T_3 were not significantly different between groups.

CHAPTER 4

General conclusions and perspectives

4.1 Fate of dietary uptake of MeHg

This thesis investigated the effects of dietary MeHg exposure on the development of *Xenopus tropicalis* tadpoles. We originally predicted that the assimilation of MeHg from food would follow three phases: 1) initial high MeHg uptake, 2) gradual MeHg assimilation, and 3) a steady state between MeHg uptake and decontamination and/or excretion. This may result in an increase in THg in tank water. Body burden analysis indicated that all tadpoles accumulated measurable levels of THg and MeHg over time, although initial increases in Hg in ng/g were followed by decreases in all groups by the end of the experiment. Different patterns emerged when we examined Hg content at the ng/tadpole level. At low levels of Hg exposure (the control group and low MeHg group) initial accumulation of Hg was followed by a steady state for MeHg and continued increases in THg content, indicating potential depuration. At a higher level of MeHg exposure, accumulation of both MeHg and THg occurred over the entire course of the experiment and no steady state was achieved.

Upon examination of the literature (Figure 4.1), our treatment levels of MeHg fall within values measured in natural aquatic environments and within the recommended limits for consumption by humans given by Health Canada and the World Health Organization. Concentrations of MeHg found in the control and 50 ng/g MeHg groups are representative of those found in numerous ecosystem components that tadpoles commonly access as food, such as phytoplankton and zooplankton. Concentrations of MeHg found in the 250 ng/g group are representative of those that would be consumed

by detrivores feeding on diet items such as fish carcasses, or as discussed in Chapter 1, diet items such as periphyton, phytoplankton, and zooplankton found in contaminated environments.

4.2 Developmental alterations

Several developmental alterations were observed in tadpoles exposed to the high MeHg treatment. Percent mortality and percent metamorphosis were elevated in this group when compared to tadpoles in the control group. Although age at metamorphosis in the high MeHg group was not greater than age in the control or low MeHg groups, the total number of days between each tadpole reaching metamorphosis was greater, as was tadpole mass and size. The combination of these alterations implies that a developmental delay was occurring as rate of development is inversely related to larval growth rate and size at metamorphosis (Denver et al. 2002). Although significant differences in T_3 levels were not found to exist between groups, this does not rule out the possibility of a developmental delay in high MeHg group tadpoles as it could have occurred prior to metamorphic climax or may have resulted from the disruption of another component of the TH axis.

Behavioural and physiological abnormalities were observed in a dose dependant manner. Motor control changes such as lethargy, resting on the bottoms and floating on the surface of the water of tanks are similar to those observed in other organisms such as mink (Dansereau et al. 1999). Increased skin pigmentation and enlarged, pigmented livers were observed in a high proportion of tadpoles from the high MeHg group and to a much lesser extent in tadpoles from the low MeHg group. It is thought that both abnormalities

are markers of toxicity (Sparling et al. 2000, Drevnick et al. 2008) and could also indicate a lack of MeHg elimination by organisms.

4.3 Significance of thesis

The major objectives of this thesis were to determine the capacity of developing tadpoles to accumulate and eliminate environmentally relevant levels of Hg and to relate developmental effects to treatment levels. To date, this is the first study to accomplish both. Although previous studies have exposed tadpoles to THg and MeHg through diet during development (Unrine et al. 2004, Gibson 2005), and have related developmental effects to treatment level, they have not fully examined these effects in relation to Hg metabolism. By measuring Hg levels in tadpoles throughout development as well as Hg concentrations in tank water, we have determined a mass balance that sheds some light onto how tadpoles accumulate, eliminate and potentially detoxify Hg.

It has been well established that amphibian populations worldwide are in decline (Blaustein and Wake 1990, Alford and Richards 1999, Houlahan et al. 2000, Stuart et al. 2004, Sodhi et al. 2008). Although Hg pollution exists on a global scale, and MeHg can be found in pristine as well as polluted environments due to its volatile inorganic nature and global transport capabilities, the potential involvement of MeHg in amphibian declines has received little attention. It is well known that MeHg can be toxic to aquatic biota by impairing productivity, growth, and development (Bank et al. 2007), and that amphibians are more susceptible to the toxic effects of contaminants than many organisms. This is because they possess a high conversion efficiency that is associated with high rates of contaminant bioaccumulation compared with other animals of similar

trophic position (Unrine et al. 2007). Although many explanations have been proposed for amphibian declines, and it is unlikely that MeHg toxicity is the sole cause, it may be a contributing factor. A high, yet still environmentally relevant level of MeHg was shown to cause increased mortality and disruption to development in tadpoles in our study. This is cause for concern as it may have important implications for conservation efforts in aquatic environments with high Hg inputs.

Although some of the mechanisms of MeHg absorption and transportation within the bodies of vertebrates have been elucidated, mechanisms of elimination during development are not well understood (Clarkson 2007). The results of the present study may have important implications for developing organisms. Amphibians are useful models for toxicological study as they share key developmental processes with all vertebrates (Tata 2006). As well, amphibians demonstrate similar hormonal responses to adverse environmental conditions as other vertebrates, such as the release of CRF in response to adverse environmental conditions (Crespi and Denver 2005). The negative developmental effects seen in the high MeHg treatment tadpoles may therefore have impacts on other organisms, even humans, as this level of exposure (250 ng/g MeHg wet wt.) is less than that recommended for MeHg consumption by Health Canada (Figure 4.1).

One goal of this thesis was to develop a technique to measure TH levels in whole body tadpoles. Relating developmental effects to disruptions in the hypothalamo-pituitary-thyroid axis is important because all of the changes that occur during larval development are a result of the actions of the components of this axis (Denver et al. 2002). By adapting techniques for TH extraction (from Denver 1993 and references

therein), we were able to successfully extract T_3 from both *X. tropicalis* and *Rana pipiens* tadpoles (in collaboration with PhD student Maxine Croteau). Moreover, we developed a technique for measuring T_3 using a commercially available EIA kit for human serum. As our TH extracts were reconstituted in human steroid-free serum from the kit manufacturer and parallelism was achieved between our extracts and the standard curve of the EIA kit, this technique has the potential for broad application across species boundaries. This new technique for measuring THs in tadpoles also offers several advantages over previous methods for TH measurement such as radio immunoassays as EIA compounds remain relatively stable for long periods of time, radioactive material is not required, and overall, EIA kits are more cost effective (Thoresen et al. 1996). To our knowledge, we are the first to measure TH levels in tadpoles using an EIA and to date no one has examined changes in T_3 levels in amphibians exposed to MeHg.

4.4 Future research directions

Additional research should be conducted to elucidate the mechanisms responsible for the observed effects of MeHg exposure. This should include chronic exposure experiments that specifically focus on Hg metabolism. It is clear that measurement of both accumulation and excretion is required to fully characterize Hg metabolism and that an accurate measurement of excretion through the sampling of feces was clearly missing from this study. Future studies should ensure that tadpole excrement is taken into account when determining Hg mass balance. Future studies could also examine the transfer of MeHg from food to tadpoles using stable isotopes of Hg, and isotopically enriched MeHg could also be used to quantify Hg distribution within tadpoles and patterns of elimination,

as has been conducted in fish (Sarica et al. 2005, Van Walleghem et al. 2007). The abundance and functions of metal detoxification proteins, such as MTs should also be investigated, especially in tissues and organs that are known to accumulate MeHg such as the brain, or those responsible for detoxification within the organism such as the liver (Clarkson et al. 2007). Knocking down MT genes in tadpoles (perhaps by morpholinos or expression of dominant-negative forms), as has been done in mice (reviewed in West et al. 2008), could help to determine if these proteins are in fact responsible for MeHg detoxification or if other protective mechanisms also exist.

Future chronic exposure studies should involve stage specific dissections to measure TH levels and gene expression. Gene expression analysis should focus on components of the TH axis such as deiodinases which have been shown to exhibit changes in relation to MeHg exposure in organisms such as fetal mice (Watanabe et al. 1999). Moreover, while not examined in this thesis, it is also possible that growth hormone (GH), prolactin (PRL) or other systems were disrupted by MeHg and could contribute to delays in metamorphosis, since GH and PRL are anti-metamorphic (Denver 1998b; see Figure 1.1). Other target genes involved in tadpole development that are not components of the TH axis, such as those controlling tissue differentiation and cell apoptosis (Monetti et al. 2002), could also be investigated using microarrays which are available for *X. tropicalis* (Chalmers et al. 2005). Finally, histological examination of the brain, liver, interrenal glands and gonads of tadpoles exposed to MeHg could provide some insight into the direct effects of MeHg on amphibian larval tissue. It could also be useful for the investigation of other effects of MeHg on development, for example sexual differentiation, because MeHg binds to ERs *in vitro* (Sukocheva et al. 2005).

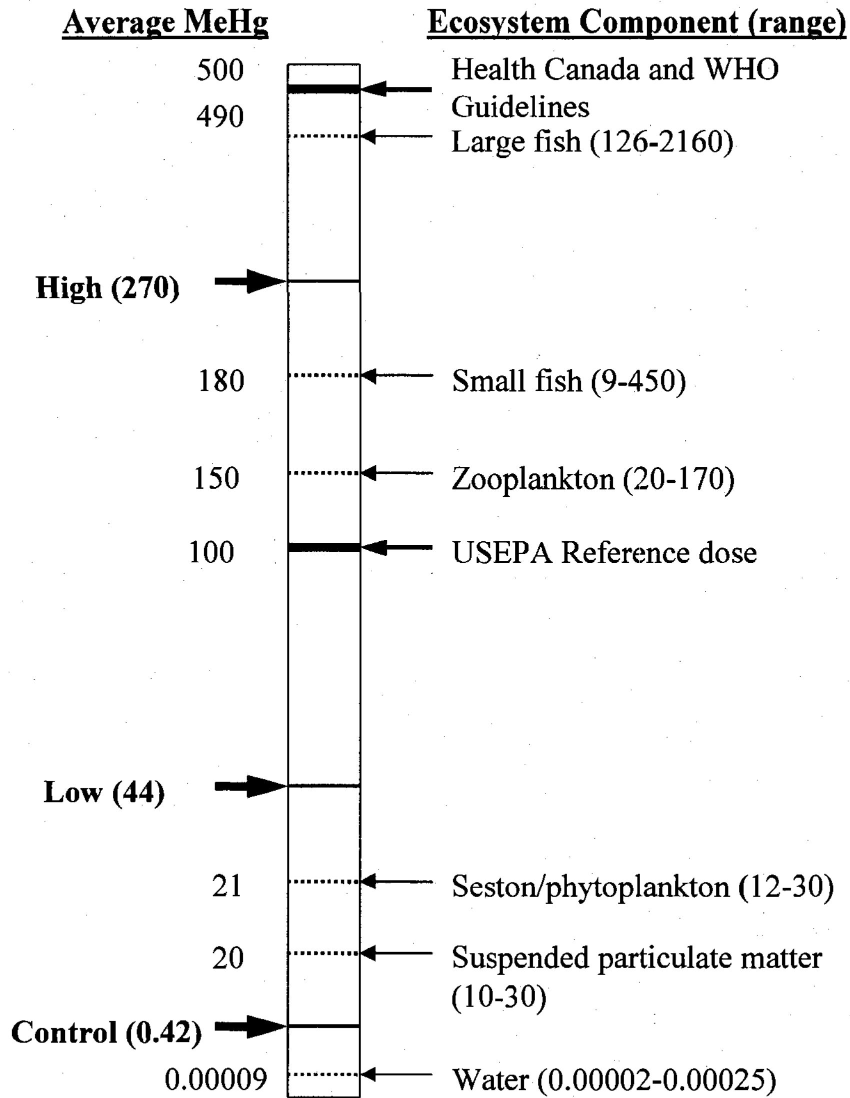


Figure 4.1. Treatment levels for the chronic exposure experiment compared to fish consumption guidelines and environmental MeHg concentrations in non-polluted freshwater systems as found in the literature. Concentrations are given on a wet weight basis. Dietary treatment and mean environmental concentrations are given on the left hand side of the ruler with ranges provided in parentheses on the right hand side. The Health Canada and World Health Organization (WHO) fish consumption guidelines are indicated by the heavy black ruling. It should be noted that the reference dose given by the United States Environmental Protection Agency (USEPA) is lower than the guidelines provided by Health Canada and the WHO. Adapted from Watras and Bloom 1992, Westcott and Kalff 1996, Hall et al. 1997, Plourde et al. 1997, Unrine and Jagoe 2004, Gibson 2005, Hickey et al. 2005 and Hammerschmid and Fitzgerald 2006.

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Appendix I – List of manuscripts not included in this thesis

Croteau M.C., **Davidson, M.A.**, Duarte, P., Popesku, J., Lean, D.R.S. and Trudeau, V.L. Assessment of thyroid system disruption in *Rana pipiens* tadpoles chronically exposed to UVB radiation and 4-tert-octylphenol. *In preparation.*

Croteau, M.C., **Davidson, M.A.**, Lean, D.R.S. and Trudeau, V.L. 2008. Linking Climate Change and Global Increases in UVB Radiation Exposure with Impacts on Amphibian Development and Metamorphosis. Physiological and Biochemical Zoology. *In press.*