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The effects of methylmercury ingestion on amphibian tadpoles

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The effects of methylmercury ingestion on amphibian tadpoles.

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Abstract

Methylmercury (MeHg) is a toxic heavy metal and a health threat to wildlife and humans, however nothing is known about its effects on amphibians. MeHg is produced from inorganic Hg in the aquatic environment, and bioaccumulates in the food chain. This exposes tadpoles to elevated levels of MeHg in their diet, and may pose a risk to development. Tadpoles of the North American species *Bufo americanus* and *Rana pipiens* as well as the African frog model species *Xenopus tropicalis* were subchronically exposed to dietary MeHg ranging in concentration from 1ng/g to 1000ng/g to determine LC50s and species sensitivity differences. A developmental differences study was also performed with *B. americanus*. The 33-day LC50 estimates indicate that Gosner stage 25 tadpoles of both *B. americanus* and *R. pipiens* were the most sensitive, and they exhibited a similar sensitivity to MeHg toxicity. The *X. tropicalis* LC50 estimate is significantly higher ($p=0.05$) than those calculated for *B. americanus* and *R. pipiens* Gosner stage 25, and the developmentally advanced *B. americanus* Gosner stage 27-30 LC50 estimate is also significantly higher ($p=0.05$) than the *B. americanus* Gosner stage 25 LC50. Behavioural changes observed in tadpoles included lethargy, inversion and emaciation, which are all attributable to MeHg poisoning as well as MeHg damage in the brain. Body burdens measured in experimental tadpoles were elevated in the MeHg treatments compared to Controls, with observed stepwise increases in body burden relative to increases in ingestion concentration. Environmental body burdens measured in tadpoles caught in Eastern Ontario and Western Quebec were comparable to experimental Control body burdens, indicated that these areas are not experiencing high levels of MeHg.

A chronic dietary MeHg exposure was performed using *X. tropicalis* tadpoles of varying developmental stages in order to investigate the effects of lower level, environmentally relevant MeHg concentrations on metamorphosis. There was an observed 140% increase in the number of tadpoles metamorphing from the low, 50ng/g MeHg treatment compared to Controls, which was determined to be a significant difference ($p<0.03$). The high, 500ng/g MeHg treatment had lower numbers of metamorphs than the Controls, and high levels of mortality compared to both Controls and the 50ng/g treatment. The most metamorphs emerged from the 50ng/g treatment when exposure began at Nieuwkoop-Faber stage 51 to 54^{+}. This study demonstrates for the first time that dietary MeHg is toxic to amphibian tadpoles, and that environmentally relevant levels of dietary MeHg can cause behavioural and developmental changes in tadpoles.

Résumé

Le méthylmercure (MeHg) est un métal extrêmement toxique pour les humains et les animaux, par contre on ignore les effets du MeHg sur les amphibiens. Le MeHg provient de la transformation du mercure inorganique dans les milieux aquatiques. Le MeHg s'accumule dans la chaîne alimentaire ce qui augmente le risque des têtards à être exposé à des concentrations élevées de MeHg dans leur diète et risque d'affecter leur développement. Les têtards d'espèces nord-américains telles que : *Bufo americanus* et *Rana pipiens*; ainsi que l'espèce africaine *Xenopus tropicalis* ont été traitées sous-chroniquement avec une alimentation contaminée au MeHg, aux concentrations variant de 1ng/g à 1000ng/g, afin de déterminer les CL50 ainsi que les différences de sensibilité entre les espèces au MeHg. Une étude a été performé pour déterminer les variations de sensibilité entre les differents stades de développement chez *B. americanus*. L'estimation de la CL50 après 33 jours indiquait que les têtards de *B. americanus* et *R. pipiens* au stade Gosner 25 sont les plus sensibles au MeHg et ont révélé une sensibilité similaire au MeHg. L'estimation de la CL50 pour les têtards de *X. tropicalis* était significativement élevée ($p=0.05$) comparer aux estimations de CL50 chez *B. americanus* et *R. pipiens*. Les têtards de *B. americanus* ont été moins sensibles au MeHg aux stades Gosner 27-30 qu'au stade Gosner 25 ($p=0.05$). Les changements de comportement observés incluaient la léthargie, l'inversion et l'amaigrissement des têtards, peuvent tous être liés à l'ingestion de MeHg et à l'endommagement du cerveau par le MeHg. Les concentrations de MeHg mesurées dans les têtards expérimentaux étaient très élevées comparativement aux contrôles. Une élévation en concentration de MeHg dans les têtards a été observée lorsqu'il y avait une augmentation de la concentration de MeHg ingéré. Les concentrations de MeHg retrouvées dans les têtards

capturés dans les marais de l'est de l'Ontario et de l'ouest du Québec sont comparables avec celles des contrôles, ce qui indique que ces régions ont des niveaux de MeHg relativement bas.

La deuxième étude soit une exposition chronique à une diète ayant des concentrations de MeHg plus faibles et environnementalement significatives, a été effectué utilisant des têtards de *X. tropicalis* à différents stades de développement. Comparativement aux contrôles, une hausse significative ($p<0.03$) de 140% a été observée dans le traitement de faible concentration, 50ng/g de MeHg. De plus, moins animaux exposés à une concentration élevée de MeHg (500ng/g) ont réussi à se métamorphoser, et leur taux de mortalité était beaucoup plus élevé que celui retrouvé dans les contrôles et dans le traitement à faible concentration. Plus les têtards avaient un stade de développement avancé lorsqu'ils étaient exposés aux traitements, plus ils atteignaient le stade de la métamorphose. Toutefois, il y a eu davantage de succès de métamorphoses dans le traitement à faible concentration de MeHg lorsque les têtards étaient exposés au stade Nieuwkoop-Faber 51 à 54^{+}. Cette étude a permis de démontrer pour la première fois que le MeHg présent dans la diète des têtards était toxique et pouvait causer des changements de comportement et de développement chez les têtards.

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List of Abbreviations

ACTH – Adrenocorticotropic hormone
AP – Anterior Pituitary
CRF – Corticotropin Releasing Factor
DCM - Dichloromethane
DI-2 – Type II Iodothyronine Deiodinase
DI-3 – Type III Iodothyronine Deiodinase
DOC – Dissolved Organic Carbon
DORM-2 – Dogfish Muscle Standard Reference Material (NRC Canada)
G – Gosner Developmental Stage
GC AFS – Gas Chromatograph Atomic Fluorescence Spectrometry
GH – Growth Hormone
HCG – Human Chorionic Gonadotropin
Hg – Mercury
HYP – Hypothalamus
LC50 – Lethal Concentration 50%
MeHg – Methylmercury
MQ – Reverse Osmosis, Milli-Q® polished water
mRNA – Messenger Ribonucleic Acid
MS-222 – Ethyl 3-aminobenzoate methanesulfonate salt
NF – Nieuwkoop-Faber Developmental Stage
PRL – Prolactin
RXR – Retinoid X Receptor
SH – Thiol functional group
T_2 – Diiodothyronine
T_3 – Triiodothyronine
T_4 – Thyroxine
totHg – Total Mercury
TR – Thyroid hormone receptor
TRE – Thyroid response element
TRH – Thyrotropin releasing hormone
TSH – Thyroid stimulating hormone
UV – Ultraviolet Radiation

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Chapter 1

1.0 The importance of amphibian sensitivity and methylmercury

1.1 Introduction

The first portion of the thesis reports results of methylmercury (MeHg) toxicity research on two native Canadian and one African amphibian species. The second portion of the thesis reports results of chronic MeHg exposure to the African species, *Xenopus tropicalis*.

The Class Amphibia is one of the oldest classes of land-dwelling organisms in evolutionary time (Dorit et al. 1991). The decline and disappearance of amphibian populations worldwide is a concern that has arisen within the past three decades. It has been recognized that there is a trend of population decline happening among amphibian species worldwide (Houlahan et al. 2000). In fact, one recent study suggests that amphibians are the most rapidly declining class of organisms today, and almost 50% of the species declines result from unknown causes (Stuart et al. 2004).

Known as environmental 'canaries in the coal mine', amphibian species are notably sensitive to changes in the environment, allowing many hypotheses to be formed to attempt to explain their disappearance. Suggested causes include acid rain deposition in pristine areas (Baraniga 1990), environmental pollution by pesticides and other persistent chemicals (Alvarez et al. 1995, Gillan et al. 1998, Herkovits et al. 1998), increasing ultraviolet (UV) radiation exposure (Blaustein et al. 1994, Ankley et al. 1998, Crump et al. 1999, Ankley et al. 2002), the introduction of exotic species (Lawler et al. 1999, Hamer et al. 2002), the increase of disease such as chytrid fungus (Beard and O'Neill 2005) and habitat fragmentation (Marsh and Pearman 1997, Pearman 1997). However, some of the declines

seen in the global amphibian population cannot be explained using any of the above concepts.

There is a paucity of information on the sensitivity of amphibians to MeHg exposure. Often categorized with other heavy metals and pollutants, MeHg stands out as it is naturally occurring, ubiquitous, transient and bioaccumulative. However, anthropogenic sources of mercury (Hg) increase the amount of Hg circulating globally and therefore the amount that is available for bioaccumulation. Mercury is released through the combustion of fossil fuels, chlor-alkali and pulp mill plant outflows and mining activities (Wiener et al. 2003). It is a global problem, as Hg can be found in pristine as well as polluted environments, due to its volatile inorganic nature and global transport capabilities. Human health concerns drives research into MeHg toxicity and global cycling because of its neurotoxicity and bioaccumulation in fish. Yet, it is of special concern for amphibians as they spend an important developmental stage captive in an environment ideal for MeHg production and transport – wetlands. This research could be considered one of the initial studies to investigate whether MeHg could play a role in amphibian declines.

In order to be able to elucidate the remaining 48% of unknown causes of global amphibian decline (Stuart et al. 2004), we must consider what physiological features and functions cause them to be such a sensitive indicator of environmental health.

1.2 Hormonal Control of Metamorphosis in Amphibians

Amphibians experience several vulnerable periods in their juvenile existence, where certain external stimuli may disrupt developmental processes and therefore reduce adult fitness. During initial embryonic development, the position of the egg masses within the

water column may affect the growth, development and survival of the embryo, depending on its exposure to UV radiation or chemical pollutants (La Clair et al. 1998, Crump et al. 1999). After hatching, the new larvae must still contend with environmental pollutants and UV radiation. Added to these stressors are intraspecific competition, predators and the possibility of habitat loss due to the drying of a pond. These factors can impact a tadpole's ability to metamorphose into the adult form by delaying or advancing metamorphosis inappropriately. Ill-timed metamorphosis can result in the juvenile being incapable of surviving in the new adult form. An example of this reduced survival is smaller size due to a short larval stage or reduced larval growth before metamorphosis, resulting in more intense adult competition due to size disadvantage (Kiesecker 1996).

Since metamorphosis is one of the most important developmental processes that tadpoles experience, it is important to review the hormones involved and how they affect tadpole development. The main neuroendocrine control feature of metamorphosis is the amphibian thyroid hormone system as controlled by the hypothalamus (Figure 1.1). The hypothalamus responds to environmental cues to modulate hormone release and metamorphic progression. Through hypothalamic control of the corticotropin releasing factor (CRF) pathway, metamorphosis is initiated and maintained. This pathway includes the release of thyroid hormones and corticosteroids. The thyrotropin releasing hormone (TRH) pathway promotes somatic growth within the tadpole by stimulating growth hormone release (Denver 1998).

CRF is the main initiator of the thyroid hormone axis of metamorphosis. It acts as a stimulator for the anterior pituitary to release adrenocorticotropic hormone (ACTH) and

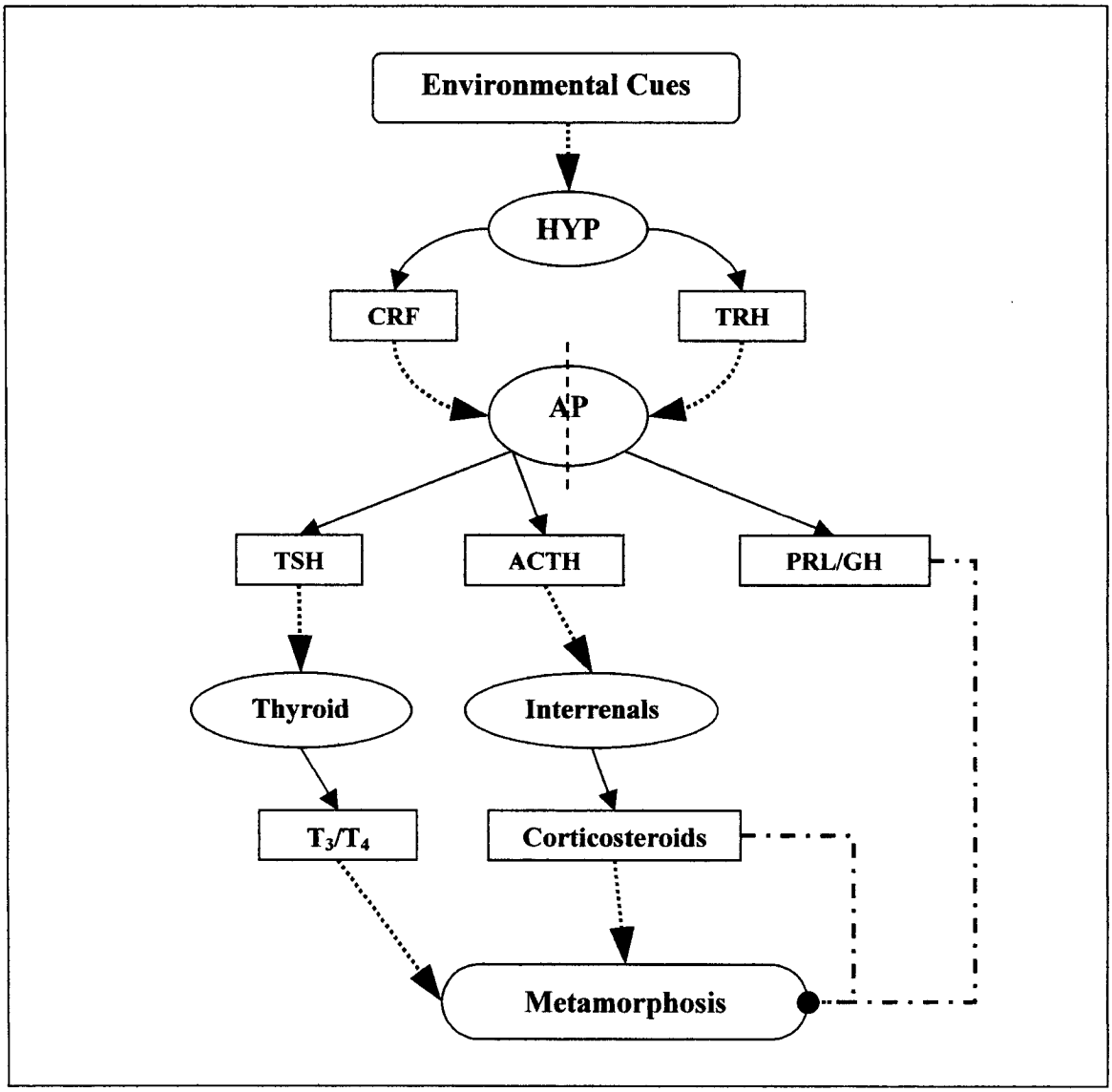

Figure 1.1 – Schematic diagram illustrating the hormonal cascade involved in amphibian metamorphosis. Dashed arrows indicate an upregulation or stimulatory influence. Dot-and-dash lines ending with a circle indicate a downregulation or inhibitory influence. Black arrows indicate a hormone product of the tissue. Dashed black lines across tissues separate cell types with differing roles. Short forms included on the diagram are as follows: ACTH – adrenocorticotropic hormone; AP – anterior pituitary; CRF – corticotropic releasing factor; GH – growth hormone; HYP – hypothalamus; PRL – prolactin; T_3 – 3-5-3' triiodothyronine; T_4 – thyroxine; TRH – thyrotropin releasing hormone; TSH – thyroid stimulating hormone. Compiled from information in Denver and Licht (1989), Hayes et al. (1993), Denver (1998), and Tata (1999).

thyroid stimulating hormone (TSH) which stimulates the release of corticosteroids from the interrenal glands and thyroid hormones, triiodothyronine (T_3) and thyroxine (T_4), from the thyroid gland, respectively (Denver and Licht 1989). Triiodothyronine is approximately 10 times more biologically active than T_4 therefore it is considered the main thyroid hormone involved with metamorphosis. Corticosteroids and thyroid hormones act synergistically to control the rate of metamorphosis (Denver 1998).

The hypothalamus-pituitary-interrenal stress axis has the ability to adjust the timing of metamorphosis to reflect the environmental stressors affecting the tadpole (Seasholtz et al. 2002). Increased corticosterone during early metamorphosis can inhibit development, whereas exposure later in metamorphosis can speed development (Hayes et al. 1993). Intraspecific competition caused increased corticosterone levels in *Rana pipiens* tadpoles, slowing both growth and development (Glennemeier and Denver 2002). Corticosterone contributes to the developmental plasticity of the desert dwelling western spadefoot toad (*Scaphiopus hammondii*), as the threat of pond desiccation promotes high CRF levels thus increasing the rate of development (Denver 1997). Developmental rates change as tadpoles respond to stress from predators, competitors and food restrictions as well (Nicieza 2000, Barnett and Richardson 2002).

A key step to trigger amphibian metamorphosis is the autoinduction of thyroid hormone receptors (TRs) by thyroid hormones. There are two genes responsible for the production of TRs, resulting in α and β subtypes. TRα is expressed from early development in *Xenopus laevis* tadpoles. TRβ is autoinduced through the increase in active T_3 during metamorphosis. These receptors are ligand-dependent transcription factors, requiring activation by thyroid hormones in order to be capable of binding to thyroid response

elements (TREs) in promoter regions of target metamorphosis-related genes. As thyroid hormone concentration increases, the synthesis of new TRβ increases the number of receptors available within the target tissues (Denver 1998, Tata 1999). It is thought that this facilitates the metamorphic changes through increased T_3-TRβ activation of other TREs to promote gene transcription (Ulisse et al. 1996). When TRs bind T_3, a dimerization of TRs begins transcription at the TREs of the controlled gene. This dimerization can result in a homodimer (two TRs) or a heterodimer, which includes the retinoid X receptor (RXR) that can also bind T_3. The heterodimerization between TR and RXR seems to be the most effective stimulator of gene transcription from the TRE (Ulisse et al. 1996, Denver 1998). Stimulation of gene transcription occurs in a tissue specific manner and involves the control of transcription factors, enzymes, proteins, and signaling molecules. This control can result in upregulation, downregulation or a biphasic response pattern in the stimulation of gene transcription depending on the metamorphic requirements. For example, the brain undergoes a widespread restructuring involving new cell division and axon growth, yet the tail and gill structures undergo programmed cell death and complete regression, while limbs and lungs require *de novo* tissue generation and innervation (Tata 1999).

Many feedback systems exist to control hormone levels. In order for T_4 to become more biologically active, one of the iodines on the molecule must be removed by type II iodothyronine deiodinase (DI-2). In this way, T_4 becomes the biologically active T_3, 3-5-3′ triiodothyronine (Huang et al. 2001). However, type III iodothyronine deiodinase (DI-3) converts T_4 to reverse-T_3, 3-5′-3′ triiodothyronine, which is not biologically active. DI-3 can also convert active T_3 into inactive diiodothyronine (T_2) (Norris 1997). The amount of biologically active thyroid hormone is regulated through these mechanisms. Additional

regulatory pathways exist to maintain a strict control over metamorphosis, including the effects of prolactin (PRL), shown to inhibit TR mRNA induction by T_3 (Tata 1999) which then inhibits metamorphosis.

The hormonal control of amphibian metamorphosis is responsible for regulated development within the larval form. If any point within this system was disrupted, the effects could be lasting, and possibly lethal. The effect of MeHg on the development of amphibians has not been previously considered, but it is known that MeHg can affect other organisms in an acute and sublethal manner.

1.3 Mercury

Amphibians are exposed to MeHg due to their habitat requirements. This includes wetlands, roadside ditches, farm ponds, man-made and natural ponds (Chapter 2) in addition to areas of industrial activity such as mining (Winch, pers. comm.). Therefore, in order to set the context of this next section of the review, a brief overview of Hg cycling and some of its chemical interactions will be presented.

1.3.1 Mercury Cycling

Mercury is a chemical of concern because it is a highly toxic heavy metal with no known biological function. It is released through natural events such as volcanic eruptions as well as from anthropogenic sources including fossil fuel combustion, chlor-alkali plants and mining operations (Wiener et al. 2003). Atmospheric Hg is usually transported globally as the volatile elemental species, Hg^0. Wet and dry deposition of Hg is generally in the oxidized form of Hg^{2+} and, to a small degree, MeHg (Porcella 1994, Morel et al. 1998).

Hg^{2+} can be methylated in forest soils (Verta et al. 1994) and wetlands (Rudd 1995). Within water bodies there is a constant flux of atomic Hg species cycling through the water column. These atomic species bind to dissolved organic carbon (DOC) or other molecules (Hintelmann et al. 1995), and are considered less biologically active. Reduction occurs through bacterial processes or photodegradation by UV radiation, returning MeHg to Hg^{2+} and Hg^0, thereby allowing volatilization (Sellers et al. 1996, Morel et al. 1998). UV also photo-oxidizes DOC, releasing bound metals back into the water column (Winch et al. 2002). Water pH can increase bioavailability, as higher acidity releases Hg species from DOC (Melamed et al. 2000, Amirbahman et al. 2002). Water pH has been found to be the greatest predictor of fish tissue Hg, in concert with DOC and MeHg levels in water (Hickey et al. 2005). Sedimentation can provide a sink for Hg species in lakes. However, Hg^{2+} becomes methylated within the sediments through the actions of sulfate-reducing bacteria (Morel et al. 1998) and may provide a large source of MeHg to the lake system (Sellers et al. 1996). It has been found that there is microbial demethylation within the sediments of freshwater systems, for example in the Florida Everglades (Martin-Dipasquale et al. 2000) although whether this acts as a major MeHg removal mechanism remains to be seen.

1.3.2 Methylmercury

Methylmercury is the most toxic and biologically active of the Hg species. It easily crosses the blood-brain (Clarkson 1994) as well as the placental barriers, facilitated by its propensity for binding to thiol (SH) groups in cysteine (Simmons-Willis et al. 2002). MeHg is the reason for the concern about bioaccumulation of Hg within the food chain (Watras et al. 1994), possibly due to the inability of many higher organisms to effectively excrete it

(Clarkson 1994). MeHg is thought to be mainly produced through the microbial processes of sulfate-reducing bacteria in sediments (Morel et al. 1998), but there have been indications of an abiotic pathway which can methylate Hg through DOC (Weber 1993, Celo et al. 2005).

Wetlands are a key source of MeHg. Often, the water concentration of MeHg can be correlated to number of wetlands within a lake's catchment area (Hurley et al. 1995, St. Louis et al. 1996). There are a number of features of wetlands which may allow this to occur. Firstly, the high organic content of wetland water transports MeHg complexed with DOC into lake systems downstream (St. Louis et al. 1994). The second wetland feature is the existence of ideal conditions for sulfate-reducing bacteria to methylate Hg within anoxic sediments of wetlands (St. Louis et al. 1994, Langer et al. 2001). Thirdly, DOC within the wetlands also protects MeHg from UV photo-oxidation by attenuating these wavelengths within the first few centimeters of the surface (Crump 2001). All of these factors cause wetlands to have ideal conditions to produce MeHg.

1.4 Effects of Mercury on Animals

Methylmercury toxicity has been previously demonstrated in many species. The aim of this section is to review research on effects of MeHg on animals to show that biological response to MeHg insult is conserved across taxa.

1.4.1 Anatomical and behavioural MeHg effects

Piscivorous wildlife species are the primary targets for anatomical studies involving MeHg. Many studies focus on mink and otter, as well as herons, loons and other piscivorous birds. Less work has been performed on fish and amphibians.

Early studies investigating the effects of dietary exposure to MeHg on mink (*Mustela vision*) showed the acute toxicity of this chemical. Within 25 days of the beginning of feeding, mink ingesting 5μg/g dry wt. were uncoordinated, unbalanced, anorexic and had lost significant body mass. Symptoms quickly grew worse, and mink experienced ataxia, paralysis, tremors and convulsions. Within 48 hours, the animals were usually dead (Aulerich et al. 1974). Mink fed 1μg/g dry wt. experienced symptoms of MeHg intoxication 3 months into a 6 month trial. Animals were lethargic, weak in the hind limbs, anorexic and experienced head tremors and convulsions before dying or being euthanized. Histological analyses of MeHg fed animals showed brain lesions, abnormalities in blood vessel anatomy, neural degeneration of the CNS parietal and frontal lobes (Wren et al. 1987) as well as hemorrhages within the lungs, enlarged heart and kidneys, and pale liver and kidneys exhibiting cellular degeneration (Aulerich et al. 1974). Body compartment MeHg analysis in wild-caught mink and otters (*Lutra canadensis*) in several pristine and historically contaminated areas of Ontario indicated that the smaller mink had significantly higher levels of MeHg in liver and kidney tissues than the larger otters. Liver usually contained the highest concentrations of Hg, followed by kidney, muscle and brain tissue for both otter and mink (Wren et al. 1986).

Behavioural, reproductive and anatomical endpoints have been investigated for birds. Male mallards (*Anas platyrhynchos*) fed a diet of 10μg/g MeHg began to experience a progressive paralysis of the legs after 7 weeks of exposure. Hatching success was significantly lower for females on the 10μg/g MeHg and the 10μg/g MeHg plus 10μg/g selenium diets, and the MeHg diet resulted in the second highest number of hydrocephaly deformities in embryos for experimental treatments (Heinz and Hoffman 1998). With

increasing liver Hg concentrations, wild-caught surf scoters (*Melanitta perspicillata*) had lower body, heart and liver masses, attributed to possible tissue atrophy as well as loss of lipid reserves. The mean liver Hg concentrations in surf scoters and greater scaup (*Aythya marila*) were 19μg/g at a site near an abandoned coastal mining operation, and 10 and 6μg/g at an industrialized area in San Francisco Bay, respectively (Hoffman et al. 1998). There is a long list of symptoms of Hg ingestion in birds that includes decreased nesting, hatching and egg laying success in the common loon (*Gavia immer*) and common tern (*Sterna hirundo*), chronic disease and emaciation in the great white heron (*Ardea herodias*), and death in many songbird and water bird species (Meyer et al. 1998, Wolfe et al. 1998).

Recently, studies have investigated the effects of MeHg on fish. Atlantic salmon (*Salmo salar*) were fed 5μg/g MeHg for four months. Histology performed on the brains of the test group revealed the most severe vacuolation occurred within the medulla, cerebellum and ventral regions of the tectum and cerebrum. Vacuolation is the development of pockets, or vacuoles, of fluid within tissue. Necrosis of white matter and pyramidal cell swelling were also observed within the medulla. The interface of the grey and white matter in the cerebrum and tectum was intensely vacuolated. Foci of necrosis and astrocyte proliferation were very prevalent within the white matter of the brain stem. Fish exposed to 10μg/g MeHg for the same time frame had more severe effects, with gross oedemous separation of the grey and white matter extending to the forebrain in 60% of fish examined. The damage was so severe the researchers had difficulty identifying the interface between regions of the brain (Berntssen et al. 2003). A single dietary dose of 0.26μg MeHg/g body wt. to immature Arctic charr (*Salvelinus alpinus*) resulted in multiple and massive necrosis of hepatocytes, and increased metabolism indicated by a rapid decrease in stored lipids within the liver.

Increased connective tissue, decreased nucleus size and the presence of phagocytes near damaged areas occurred within 18 days of exposure. Tissue recovery occurred 30 days after dosing, but areas of necrosis still existed within liver tissues (Oliveira Ribeiro et al. 2002).

A study by Chang et al. (1974) investigated the effects of MeHg in water on *R. pipiens* tadpoles late in metamorphosis, using very elevated water concentrations. Tadpoles in 0.05 to 0.1mg/L MeHgCl displayed abnormal swimming postures, difficulties breathing and distension of the body due to fluid buildup within body cavities, before 100% mortality was reached in 48 hours. Those in the 0.5 to 1mg/L MeHgCl also displayed these symptoms, as well as attempts to escape the tank, before 100% mortality was reached at 24 hours. Within the lowest concentrations, 0.001 to 0.01mg/L, lethargy appeared within 24 hours of exposure, and metamorphosis arrested at the present stage despite continued survival for 3 to 4 more months. A second experiment involved tadpoles kept in clean water being injected five times with MeHgCl for a dosage of 0.025mg Hg per injection over ten days. These tadpoles experienced oedema of the hind legs which caused them to swell 3 to 5 times normal size. Muscle fibres were also seen to have separated due to the fluid buildup between the skin and underlying muscle tissue. A buildup of blood pigments, hemosiderin and lipofuscin, was found in the livers of these tadpoles which was thought to be the result of haemolysis of red blood cells. Interrenal necrosis and hyperchromatic, hemopoietic cells within renal tubules were also found. This study design is flawed in terms of environmental relevance as such extremely elevated levels of MeHg in water are not likely to be found in tadpole habitats where concentrations are on the order of ng/L.

A more recent study by Unrine et al. (2004) investigated the effects of low level dietary MeHg exposure in *R. sphenocephala* tadpoles. They found that the most rapid

development occurred within the medium and high treatment groups, corresponding to approximately 27 and 49ng/g MeHg dietary exposure, respectively. Days post-hatch to full hind limb development and forelimb development was significantly shorter in the high treatment than controls, and mean tadpole mass 55 days post hatch was significantly higher in the high treatment group compared to controls. Malformation of tadpoles was seen in MeHg treated tadpoles, including concentration dependent mild to severe scoliosis, as well as two tadpoles with limb deformities (micromelia, ectromelia).

1.4.2 Cellular, biochemical and genetic effects of MeHg

Studies on sublethal MeHg effects have elucidated some of the cellular and biochemical effects of MeHg exposure. Many of these studies have been conducted using *in vitro* cell lines to show cellular effects of MeHg exposure, but *in vivo* experimentation has demonstrated several key gene transcription and biochemical effects.

Microtubules are the cellular components which experience the most trauma when MeHg exposure occurs. Microtubules are associated with nuclear division, as well as movement of organelles and neural organization therefore the breakdown of microtubule function will lead to the inability of the cell to perform these processes. In numerous studies involving different cell lines, it has been shown that microtubules are disrupted by MeHg exposure (Braeckman and Raes 1999). Microtubules are disassembled (Graff et al. 1997), and depolymerized in such a way to create insoluble aggregates of microtubule subunits (Hunter and Brown 2000). After MeHg insult, microtubules become non-functional and cannot be rebuilt.

Small aggregates of chromosomal material outside of regular, functional nuclei, called micronuclei, have been induced in beluga whale (*Delphinapterus leucas*) skin fibroblasts by the application of 0.05mg/L MeHg (Gauthier et al. 1998). The formation of micronuclei is usually associated with chromosomal aberration and breakage. Structural and numerical chromosomal aberrations, like hyperdiploidy and polyploidy, were induced in human lymphocytes in a dose-dependent manner from 0.03mg/L to 6.2mg/L MeHgCl solutions (Betti et al. 1992). Fetal rat (*Rattus norvegicus*) central nervous system cells exposed to 0.46mg/L MeHgOH resulted in cells incapable of entering mitosis, whereas the 0.93mg/L level resulted in the inhibition of any cell cycling (Ponce et al. 1994). This cell cycling inhibition could be associated with microtubule failure, resulting in the inability to complete cell processes such as chromosome separation.

Rat cerebellar granule cells exposed to 2.3mg/L MeHgOH experienced acute neuronal necrosis within one hour. At the 0.23mg/L MeHgOH treatment level, a longer exposure of 9 hours resulted in 100% apoptotic cell death. This cell death was not prevented by the application of taxol, a microtubule stabilizer. A decrease in mitochondrial function was also observed in both treatments, through the loss of mitochondrial membrane potential (Castoldi et al. 2000). Mitochondria in an insect cell line (*Aedes albopictus*) became swollen after 1.18mg/L MeHg treatment, and began to show signs of necrosis (Braeckman and Raes 1999). Mitochondrial function was inhibited in *R. pipiens* motor nerve cells when these cells were exposed to 25mg/L MeHgCl through the dissipation of the mitochondrial membrane potential and release of Ca^{2+}. Mitochondrial release of Ca^{2+} was measurable within 10 minutes of direct MeHg exposure (Provan and Miyamoto 1995).

Briefly, MeHgCl has also been shown to affect gene transcription in *X. laevis* embryos. The complete inhibition of two gene fragments was achieved through the application of 0.06mg/L MeHgCl to embryos. Genes were viewed by differential display. One gene was identified as a fragment of a potential homeodomain-interacting protein kinase 3 (HIPK3). In humans, HIPK3 is a serine/threonine kinase responsible for the repression of the transcription of genes involved in developmental processes like organogenesis and apoptosis. The second gene was similar to the human iron-sulfur subunit of succinate dehydrogenase, which is involved in metabolism and cellular respiration (Monetti et al. 2002). The complete inhibition of these genes in the embryonic development of *X. laevis* could affect the survival of embryos by interfering with organ development and cellular respiration.

Several studies have investigated hormonal effects after MeHg application. The *in utero* exposure of mice (*Mus musculus*) to MeHg injections of 3μg/g body wt. for three days resulted in the depression of 5-iodothyronine deiodinase (DI-2) and elevation of 5'-iodothyronine deiodinase (DI-3) in the brains of fetal mice compared to controls. The activity of DI-2 was also significantly elevated in the placenta in both the low (one injection of 5μg/g body wt.) and high (3 injections of 3μg/g body wt.) treatments (Watanabe et al. 1999). The reduced gonadal development and reproductive success in female fathead minnows (*Pimephales promelas*) was associated with MeHg at dietary levels of 0.88μg/g to 8.46μg/g MeHg (Hammerschmidt et al. 2002). Researchers believed that MeHg interfered in the production of estrogen. Friedmann et al. (1996) found that MeHg ingestion by juvenile walleye (*Stizostedion vitreum*) caused testicular atrophy. Friedmann et al. (2002) also found a significant trend of decreasing 11-ketotestosterone with increasing Hg body

burden in largemouth bass (*Micropterus salmoides*). It has recently been shown that MeHg binds to estrogen receptor α and inhibits ligand binding *in vitro* in a human breast cancer cell line. This binding to the receptor may also promote gene transcription at estrogen response elements (Martin et al. 2003).

Physiological, histological, hormonal and genetic studies have all revealed sublethal modes of action of MeHg exposure. All taxa are affected by MeHg. Acute toxicity from MeHg poisoning has been recognized for many years. It is the low level exposure causing changes in essential systems like hormonal control of thyroid and sex steroids, potential changes in metabolism through the effect on mitochondria, and the ability to affect gene transcription during development which is novel and increasingly important. Mercury is a global pollutant, released through natural and anthropogenic activities, being transformed to MeHg in natural systems and bioaccumulating in the food chain. If low level exposure can elicit a sublethal response on body systems such as hormonal control, it is important to research MeHg effects on amphibians.

1.5 Rationale, Hypotheses and Objectives

The effects of MeHg are wide ranging and destructive in the species studied to date, but amphibians may be especially sensitive to the physiologically disrupting effects of MeHg. The evidence that mice prenatally exposed to MeHg incur changes to the iodothyronine deiodinases within the brain (Watanabe et al. 1999) indicates that MeHg may be able to modulate the levels of DI-2 and DI-3 during development in tadpole metamorphosis. A resulting imbalance in thyroid hormones in the metamorphic pathway (Figure 1.1) could change the rate of metamorphosis, which could lead to death. Damage to

microtubules by MeHg can also severely impact tadpole metamorphosis as this is a time of intense cellular change, which requires cell division and neuronal migration (Tata 1999). Additionally, the potential stress placed on developing tadpoles by MeHg could cause metamorphosis to occur at a faster rate, resulting in smaller juveniles less capable of surviving.

Few studies have investigated the challenges facing amphibians by the threat of MeHg in the environment, and those that have, have used unrealistically high water concentrations to estimate toxicity (e.g. Chang et al. 1974). Throughout embryological development and metamorphosis, amphibians are captive in a high MeHg environment. It is possible that MeHg could play a part in the global reduced survivorship of amphibians, by increasing the stress that affects developing tadpoles and potentially affecting the hormonal and cellular processes of development.

My study focused on the effects of MeHg ingestion on amphibian metamorphosis through the larval period, including toxicity. Although amphibians pass through two vulnerable life stages when confined to the water, only the free swimming larval stage was investigated in this study. Ingestion of MeHg is the environmentally relevant exposure route (Hall et al. 1997, Unrine et al. 2004), rather than exposure through elevated concentrations in water that was used by others (Chang et al. 1974, Linder and Grillitsch 2000), but is less likely to be seen in the environment.

I hypothesized that MeHg has an effect on amphibian metamorphosis, and predicted that this effect would have two phases. Low level MeHg ingestion should cause an increase in the rate of metamorphosis. At high treatment levels, it was predicted that MeHg ingestion would inhibit metamorphosis, resulting in low metamorphic rates and high mortality rates.

Additionally, changes in growth, behaviour, and body morphology were predicted to be dependent on the treatment level of MeHg ingestion. It was also expected that MeHg would bioaccumulate in the tadpoles. Lastly, differences in species sensitivity to MeHg were expected to be seen within the lethal concentration 50% (LC50) estimates as well as other measured or observed endpoints.

The objectives of this study were 1) to observe the subchronic effects of MeHg dietary exposure on three species of amphibian tadpoles using toxicity as an endpoint; 2) to determine the chronic effects of MeHg dietary exposure on the metamorphosis of the laboratory model species *Xenopus (Silurana) tropicalis*; 3) to investigate the relationship of MeHg ingestion to the relative body burdens of MeHg in the tadpoles; 4) to relate the effects seen in the laboratory to the environmental levels measured at several pristine ponds in Eastern Ontario and Western Quebec. 'Subchronic' was defined as a longer exposure period than a traditional acute toxicity test but shorter than a chronic exposure, using dietary MeHg levels high enough to induce toxicity, and the defined endpoint being 100% mortality in the highest concentration, rather than a set time period. This was chosen due to the high, environmentally unrealistic levels required to produce a reaction within the time constraints of an acute exposure. 'Chronic' was defined as an exposure period affecting a significant portion of an animal's developmental period (i.e. metamorphosis) with daily exposure to dietary MeHg and no significant death occurring during the first seven days of experimentation.

Chapter 2

2.0 Methylmercury toxicity to amphibian tadpoles: a subchronic study

2.1 Introduction

Most MeHg studies focus on piscivorous wildlife and fish, especially sport fish consumed by humans. These studies are important because of the health risk the MeHg poses to humans. However, there are clear gaps in our scientific knowledge of MeHg. The goal of this section is to examine the toxicity of dietary MeHg to amphibians and to identify species differences in MeHg effects. Body burdens were measured to compare experimental animals to tadpole samples from the natural environment. Subchronic was defined as a longer exposure period than a traditional acute toxicity test using MeHg levels high enough to induce toxicity, with the defined endpoint being 100% mortality in the highest concentration, rather than a set time period.

Previous LC50s have been performed with amphibian embryos and tadpoles, but many of these experiments were exposures to inorganic HgCl in water. For example, out of 51 cited LC50s for amphibians, 48 were based on HgCl exposure, one was an exposure to phenyl mercury acetate and two were based on MeHgCl presence in water (Linder and Grillitsch 2000). Since the exposure pathway is more likely to be through food consumption, experiments such as these have little environmental relevance, especially at high concentrations of MeHg in water. Concentrations of MeHg in natural waters are usually far lower than when it biomagnifies within the food chain. Hall et al. (1997) have stated that the mode of MeHg uptake in fish is through the food chain, and that MeHg absorption from the water column through the gills accounts for less than 15% of MeHg accumulation. We suggest that tadpoles also ingest more MeHg than they can absorb from

the water column. Consequently, establishing LC50s based on dietary MeHg is more environmentally relevant than those based on unrealistically high water concentrations.

Many toxicity studies also involve extremely short exposure periods of 24 to 96 hours. Although this generates data at a consistent time interval, these acute toxicity measurements do not reflect environmental conditions where organisms are exposed to toxic chemicals for longer time periods, e.g. a growing season. A subchronic exposure should be longer than the typical acute toxicity study and allows for observation of sublethal effects. Furthermore, lower levels of exposure which are more reflective of natural environmental contamination can be investigated. Recently, Unrine et al. (2004) performed a chronic dietary MeHg study showing effects on metamorphic rate and body weight using *Rana sphenocephala*. The present study was initiated before the Unrine et al. (2004) paper was published. Although observations are complimentary, many additional observations were made in the present study.

Wetlands are a source of MeHg to other water systems (Rudd 1995), and are the main breeding sites for amphibians. North American tadpoles will spend between two months to just over one year growing and developing within wetland systems (Behler and King 2002). During these months, they are herbivorous and detrivorous. An overview of values in the literature for environmental concentrations of MeHg is presented in Figure 2.1. Due to the variability of food sources, tadpoles could potentially graze at several levels in this schematic. As detrivores, tadpoles would eat any material, such as dead zooplankton, fish or plant detritus, all of which could provide a source of MeHg to the tadpole. As herbivores, tadpoles could graze upon periphyton (Unrine and Jagoe 2004). Tadpoles are

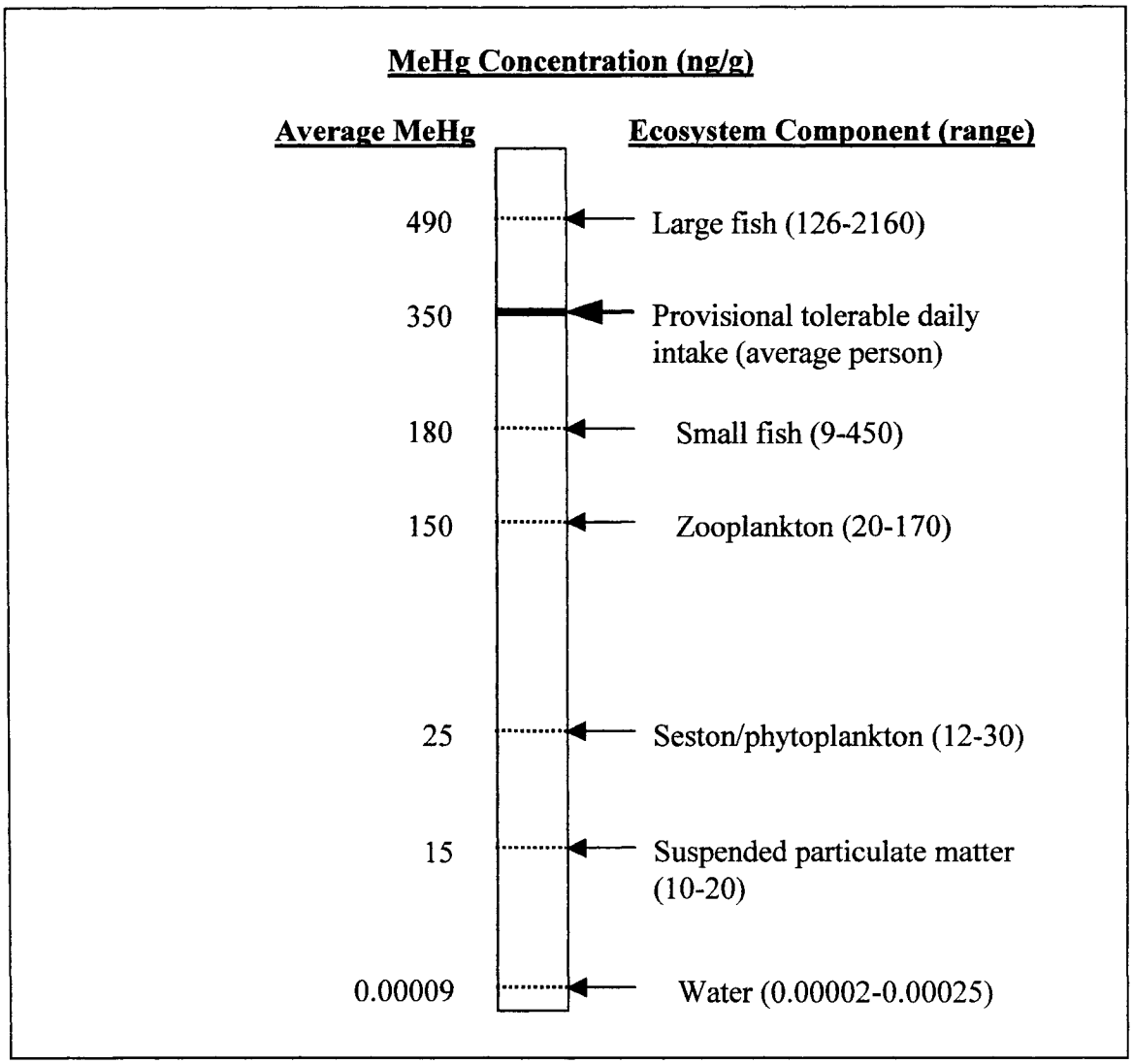

Figure 2.1 – General schematic of environmental MeHg concentrations for non-polluted freshwater systems as found in the literature. All concentrations are given on a dry weight basis, where applicable. Mean environmental concentrations are given on the left hand side of the ruler with ranges provided in parentheses on the right hand side of the ruler. A calculated daily consumption guideline is indicated by the heavy black ruling, adapted from the provisional tolerable weekly intake published by JECFA (2003). Adapted from Hall et al. (1997), Hickey et al. (2005), Plourde et al. (1997), Unrine and Jagoe (2004), Watras and Bloom (1992), Westcott and Kalff (1996), Wren et al. (1986).

cannibalistic and will also eat conspecifics, whether dead, slow or injured, thus MeHg accumulated by prey tadpoles will be transferred to the predator tadpole (Gibson, pers. obs.). Any potential food source within aquatic systems has the ability to carry MeHg which tadpoles will ingest and possibly bioaccumulate. Thus, studies involving the ingestion of MeHg are crucial to discover the environmental consequences of tadpole developmental exposure to constant dietary MeHg.

Sensitivities differ between amphibian species for chemical exposures, including acidity (Kiesecker 1996). It is likely that different amphibian species would respond differently to toxic chemicals such as MeHg as well. Therefore, it is important to investigate several species to ensure that the most sensitive response is identified. Protecting the most sensitive species will also protect those less sensitive. It is also important to identify stage specific sensitivities when a chemical could pose a greater threat to survival and reproduction. In this study, two subchronic toxicity tests were performed on one native amphibian species, where exposure began at different developmental stages.

The purpose of this study was to determine whether species differences exist in MeHg sensitivity and to develop toxicity measurements for two species of native amphibians, *R. pipiens* and *Bufo americanus*, and one non-native lab model amphibian, *Xenopus (Silurana) tropicalis*. These amphibians belong to three different Anuran families: Ranidae, Bufonidae and Pipidae, which allows this study to investigate three evolutionarily distinct amphibian species. It was hypothesized that species differences exist in MeHg sensitivity. It was also predicted that MeHg ingestion would cause changes in behaviour and body morphology, as MeHg poisoning begins to affect tadpoles. Body burden analyses were

also carried out to examine the accumulation of MeHg over time and compare these values to natural populations.

2.2 Methods

2.2.1 Tadpole collection

All native species of tadpoles were gathered from the Eastern Ontario and Western Quebec area from water bodies with no known anthropogenic MeHg contamination. *Bufo americanus* tadpoles were gathered in July 2003 from a small farm pond (20mx20m) in a fallow field outside of Cornwall, Ontario. Tadpoles were transported and acclimated to indoor conditions in opaque plastic pails in an aerated mixture of pond water and dechloraminated system water commonly used in the Aquatic Animal Care facility at the University of Ottawa. Early stage *B. americanus* tadpoles were gathered in May 2004 from a beaver-dammed, seep-fed lake (100mx120m) in the Gatineau Hills outside of Wakefield, Quebec. Tadpoles were transported and acclimated to indoor conditions in opaque plastic pails in lake water which was aerated with a bubbler. Both sets of tadpoles were verified for species (Berrill, 2002) and transferred to glass aquaria filled with room temperature, aerated system water. Tadpoles were fed Nasco® tadpole granules supplemented with Nutrafin® fish flakes until treatments began.

Collections of *Rana pipiens* partial egg masses occurred in May 2004 from a former sand quarry now filled with water (60mx120m) in Chelsea, Quebec. Eggs were separated and clutches mixed. Hatching took place in glass aquaria filled with room temperature, aerated system water. Tadpole densities were adjusted post hatch and tadpoles used at

Gosner stage 25 (Gosner 1960). Later analysis of some of these wild caught tadpoles showed non-detectable levels of MeHg before experimentation.

Xenopus (*Silurana*) *tropicalis* tadpoles were bred in-house. Adults were given subcutaneous priming injections of 50μL of 250unit/mL Human Chorionic Gonadotropin (HCG) and kept separated in single sex tanks overnight in Aquatic Care system water adjusted with 1.0N HCl to a pH of 5.8-6.0. Approximately 24 hours after the priming injection, a second injection of 250μL HCG was administered and breeding pairs were placed in clean system water at a pH of 5.8-6.0. Tanks were shaded with black plastic to prevent disturbance. Within 3 hours, clasping occurred and within 5 to 6 hours of the boosting injection, eggs were netted from the breeding tanks and placed into Petri dishes at a density of 100 eggs per dish. All breeding and rearing occurred at a temperature of 24°C. Each dish contained a solution of 1:9 Modified Ringer's Solution diluted in system water with 50μg/mL gentomycin to prevent bacterial growth. Eggs hatched overnight and dead or deformed embryos were removed from the dishes. The solution in the dishes was refreshed daily. Gentomycin treatment was stopped three days post hatch, and Ringer's solution concentration was adjusted to a 1:20 ratio five days post hatch. Tadpoles reached Nieuwkoop-Faber stage 47 (Nieuwkoop and Faber 1994) five to seven days post hatch and were fed Sera Micron® powdered algae food as needed. Solutions were changed daily and dishes inspected for dead or deformed tadpoles. Tadpole densities were reduced as growth occurred. Ringer's solution was discontinued 10 days post hatch and tadpoles were transferred to glass bowls or aquaria of system water, lightly aerated, as growth continued. Tadpoles were used at different developmental stages, and were maintained in these

conditions until experimentation occurred. Developmental stage equivalency between Gosner and Nieuwkoop Faber staging systems is presented in Table 2.1.

Collections of environmental samples for MeHg analysis were taken during the field season from several ponds and lakes in Eastern Ontario and Western Quebec, including ponds where experimental tadpoles were gathered. Tadpoles were netted from the water with a clean, soft aquarium net and euthanized with ethyl 3-aminobenzoate methanesulfonate salt (MS-222). They were transported on ice back to the laboratory in clean 50mL Falcon tubes and kept at -20°C. They were washed with reverse osmosis, Milli-Q polished (MQ) water, species were identified (Berrill, 2002) and wet weight taken. Tadpoles were then freeze dried for MeHg analysis. Suspended particulate matter was collected with a drag net and emptied into 50mL clean Falcon tubes. Periphyton, algae, and insects were collected where possible and kept in 50mL clean Falcon tubes. All materials were frozen at -20°C and freeze dried for MeHg analysis.

2.2.2 Experimentation

Tadpole developmental stage was identified before tadpoles were sorted into treatment bowls (Gosner 1960, Nieuwkoop and Faber 1994), resulting in exposures beginning at Gosner stage 25 (G25) for *B. americanus* and *R. pipiens*, Gosner stages 27-30 (G27) for *B. americanus* and Nieuwkoop Faber stage 47 (NF47) for *X. tropicalis*.

Experimentation took place in 1.5L glass bowls, in a controlled environment chamber (12:12 photoperiod) using dechloraminated system water. *B. americanus* and *R. pipiens* were kept at 21°C ± 2°C and *X. tropicalis* was kept at 24°C ± 2°C. Water was circumneutral (pH 7-7.4) with a conductivity of 158.6µS and a hardness of 30-40mg/L

Table 2.1 – Equivalency table between staging systems for free swimming larval *Xenopus tropicalis* and *Rana pipiens*. Nieuwkoop-Faber stage 46 is the beginning of feeding and lasts only 1 day. Gosner stage 25 is also the beginning of feeding. Adapted from Nieuwkoop and Faber (1994) and Gosner (1960).

Stage	
Nieuwkoop-Faber	**Gosner**
46	25
47	25-27
48	
49	28
50	
51	29-30
52	31
53	32
54	34
55	36
56	38
57	39-40
58	40
59	
60	41
61	
62	
63	42-43
64	44
65	45
66	46

$CaCO_3$. MeHg content of the water was below the detection limit (<0.02ng/L). Animals were maintained at a maximum density of 1-2g/L according to tadpole mass, averaging 5-7 animals per bowl. Water was changed daily, six days a week, either a partial or complete water change. Complete washing of bowls occurred at the outset of the experimentation and as needed during experimentation to minimize handling stress in the animals. Bowls were covered with Mylar-D™, donated by Dupont, to prevent UV-B wavelengths from the lighting from interfering with the experiment. Small vent holes were cut in the Mylar to allow air circulation.

Preliminary testing validated the experimental design for delivery of dietary MeHg. A pilot study was run using a food slurry MeHg delivery system. Water testing showed negligible amounts of MeHg in the waste water from experimental bowls compared to food concentrations. Particulate filtered from waste water samples and fecal samples showed higher levels of MeHg within the target range (Appendix 1).

Food preparation involved the addition of MeHgCl to tadpole food. For *B. americanus* and *R. pipiens*, Falcon tubes were filled with deionized water and 1g of Nasco® tadpole granules for a food density of 0.02g/mL dry wt., as determined through Trudeau lab amphibian care guidelines (Croteau, pers. comm.). MeHgCl was added to the food slurry to give final concentrations of 0, 1, 10, 100 and 1000ng/g. For *X. tropicalis* food, 1g of Sera Micron® was added to deionized water for a food density of 0.02g/mL. *X. tropicalis* final MeHg food concentrations were 0, 50, 100, 500 and 1000ng/g. Food was delivered using a 5mL latex-free syringe, and each bowl received 2mL of food daily.

Tadpoles were inspected daily. Mortalities were removed and physical abnormalities and behavioural changes were noted. The main abnormalities were inversion, which

appeared as a tadpole floating, swimming or resting with its ventral side up; lethargy, a lack of movement or swimming behaviour and the requirement for a startle response test to encourage movement; bloating, a general distension of the abdominal cavity which included oedema; lumps, specific regional distension of the abdominal cavity; lordosis, a curvature of the spine beginning at the tadpole body; kinks, curvature or acute angles of the spine beginning within the tail; and emaciation, a drastic reduction in body mass compared to tadpoles within the same bowl or in other bowls, in the case of all tadpoles being emaciated within one bowl, most often recognized by a distinct loss of roundness within the abdominal cavity. Testing for lethargy involved first a visual inspection for swimming behaviour. If no swimming was observed and a lack of general movement was evident, a startle response test was applied through gentle prodding of the tail with a disposable plastic pipette. Abnormalities were noted by number of observed tadpoles with the abnormality within a bowl as well as descriptively.

Experiments were not stopped at a specific day. Instead, a biological endpoint was chosen. Once 100% mortality was reached in the highest concentration (1000ng/g) test group, the experiment was terminated and the remaining tadpoles were euthanized using MS222 at a concentration of 1g/L, massed for wet weight, washed with MQ water and freeze dried for MeHg body burden analysis. All samples were kept at -20°C until MeHg analysis was done.

2.2.3 Methylmercury Analysis

All water samples were analyzed for MeHg following the protocol of Cai et al. (1996). Tissue samples were analyzed for MeHg as described in Cai et al. (1997) with a few

modifications. Briefly, one to two freeze dried tadpoles were weighed in a 20mL glass scintillation vial, 2mL of 6N KOH and 2mL of MQ water were added to the vials and they were shaken for 4 hours at 330rpm. After shaking, 2mL of 6N HCl and 4mL of 2:1 acidic KBr:$CuSO_4$ solution were added to vials. Dichloromethane (DCM) was added by mass to approximately 6.0 to 6.5g, and vials were shaken overnight at 330rpm. The following day, samples were centrifuged at 3000rpm for 5 minutes in a Beckman J2-MC Centrifuge, and approximately half of the DCM, by mass, was extracted into 7mL glass scintillation vials containing 1mL of 0.01M $Na_2S_2O_3$. These samples were shaken for 20 minutes at 330rpm, vortexed for 30 seconds then centrifuged at 3500rpm for 5 minutes. A volume of 400µL of the $Na_2S_2O_3$ layer was extracted into 2mL microcentrifuge tubes containing 300µL of 2:1 acidic KBr:$CuSO_4$ solution. A volume of 400µL of DCM was added to the tubes and they were shaken for 15 minutes, vortexed for 15 seconds and centrifuged in an Eppendorf microcentrifuge for 30 seconds. The DCM bubble was then extracted and passed through a pipette tip packed with dried anhydrous Na_2SO_4 into low volume glass inserts in 2mL gas chromatography vials. Samples were kept sealed in the freezer until analysis by gas chromatography atomic fluorescence spectroscopy (GC AFS). Two standard reference material (DORM 2) vials were run with each 40 sample digestion. Blanks and replicates were run within digestion racks as well.

During GC AFS analysis, certain sample injection volumes were modified from the standard 5µL injection to account for high MeHg concentrations. The lowest injection volume, 0.2µL, was used for tadpoles from the 1000ng/g concentrations and DORM 2 samples. Other injection volumes of 1 and 2µL were also used for intermediate concentrations (500ng/g, 100ng/g, 50ng/g, 10ng/g) when necessary.

Statistical analyses were performed with Microsoft Excel and by hand. Estimates of LC50s were performed using the US EPA Trimmed Spearman-Karber method computer program version 1.5.

2.3 Results

Four subchronic toxicity experiments were completed: two with *B. americanus*, one with *R. pipiens* and one with *X. tropicalis*. Complete mortality was reached in the highest level (1000ng/g) for each toxicity study.

2.3.1 *Bufo americanus*

2.3.1.1 G25 Experiment

Cumulative total mortality over the course of the experiment is shown in Figure 2.2A. The mortality rates were all below 10% until day 8 when the 1000ng/g treatment mortality increased beyond the other treatments. The other treatment groups experienced less than 10% mortality throughout the experiment.

2.3.1.2 G27 Experiment

Cumulative total mortality over the course of the experiment is shown in Figure 2.2B, compared with that from the G25 experiment. The mortality rates all remained within 10% until day 25 when the death rate in the 1000ng/g treatment increased. Only the 10ng/g and the 1000ng/g treatments had over 10% mortality.

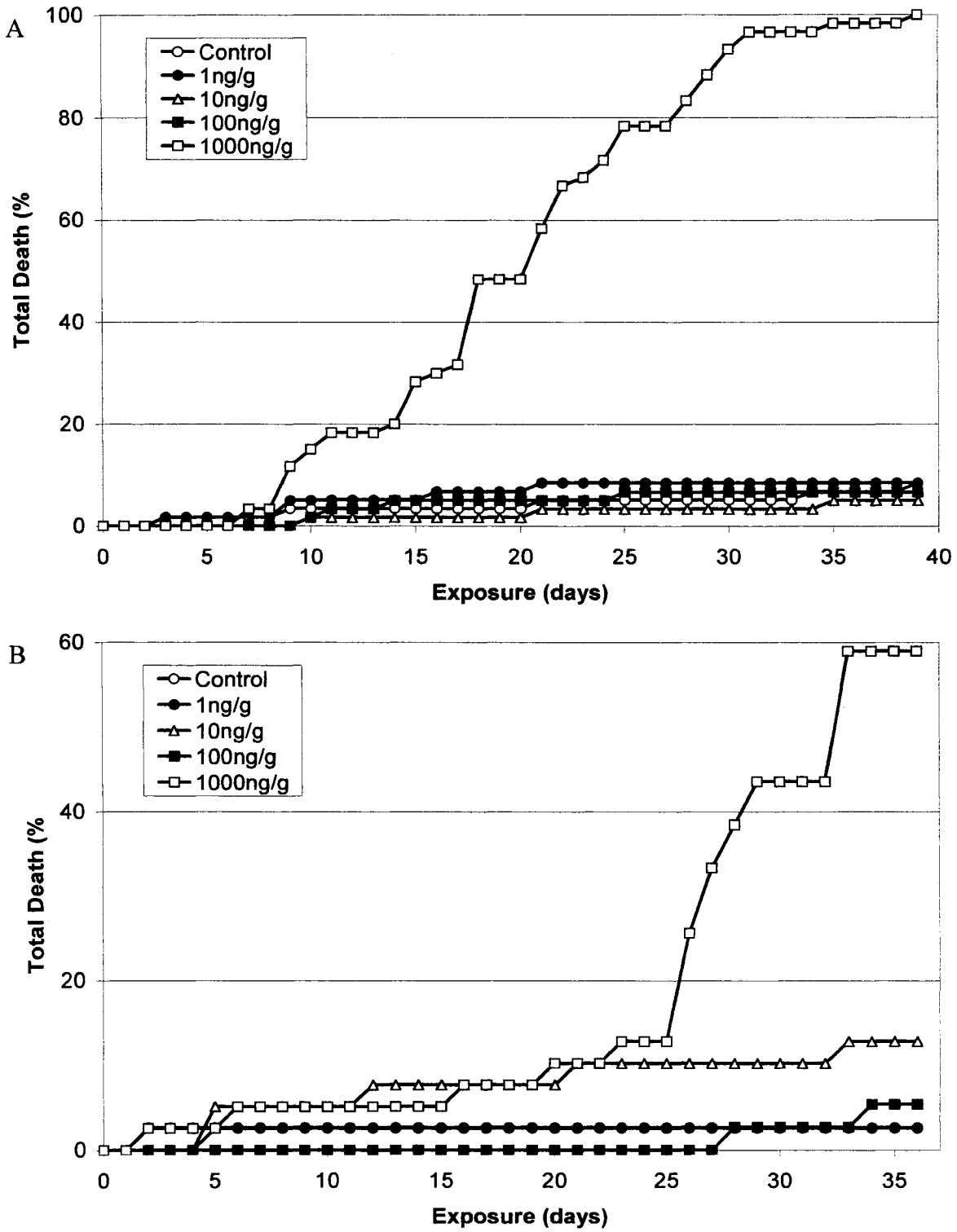

Figure 2.2 – Mortality over time in *B. americanus* MeHg toxicity studies. A) Gosner stage 25 tadpoles, n=298. B) Gosner stage 27 to 30 tadpoles, n=192. Control mortality follows the 1ng/g treatment mortality, but is obscured.

2.3.2 *Rana pipiens*

Total death in all treatments remained below 10% until day 7 when mortality increased in the 1000ng/g test level. All other treatment levels experienced low death counts with the 100ng/g treatment reaching 13% (Figure 2.3).

2.3.3 *Xenopus tropicalis*

The death profile is similar to that of the *B. americanus* G27 experiment. Cumulative total mortality remained below 10% until day 18-20 when the 1000ng/g mortality rate increased (Figure 2.4). All other treatment mortality remained below 10%.

2.3.4 Species comparisons

2.3.4.1 LC50s

By analyzing the 95% confidence limit overlap on LC50s calculated for day 33 data, the sensitivity of *B. americanus* G25 is significantly higher ($p=0.05$) than that of *B. americanus* G27 (Table 2.2).

Again, through the examination of 95% confidence limit overlap for the early developmental stage studies, it is seen that *B. americanus* G25 and *R. pipiens* G25 possess similar sensitivity to MeHg. *X. tropicalis* NF47 is shown to be significantly less sensitive to MeHg compared to the G25 experiments for native amphibian species ($p=0.05$).

The sensitivity of these species changed over the course of the experimental exposure (Figure 2.5). Early in the exposure period, it was not possible to calculate LC50 estimates from the data because not enough mortality had occurred. Throughout the remainder of the

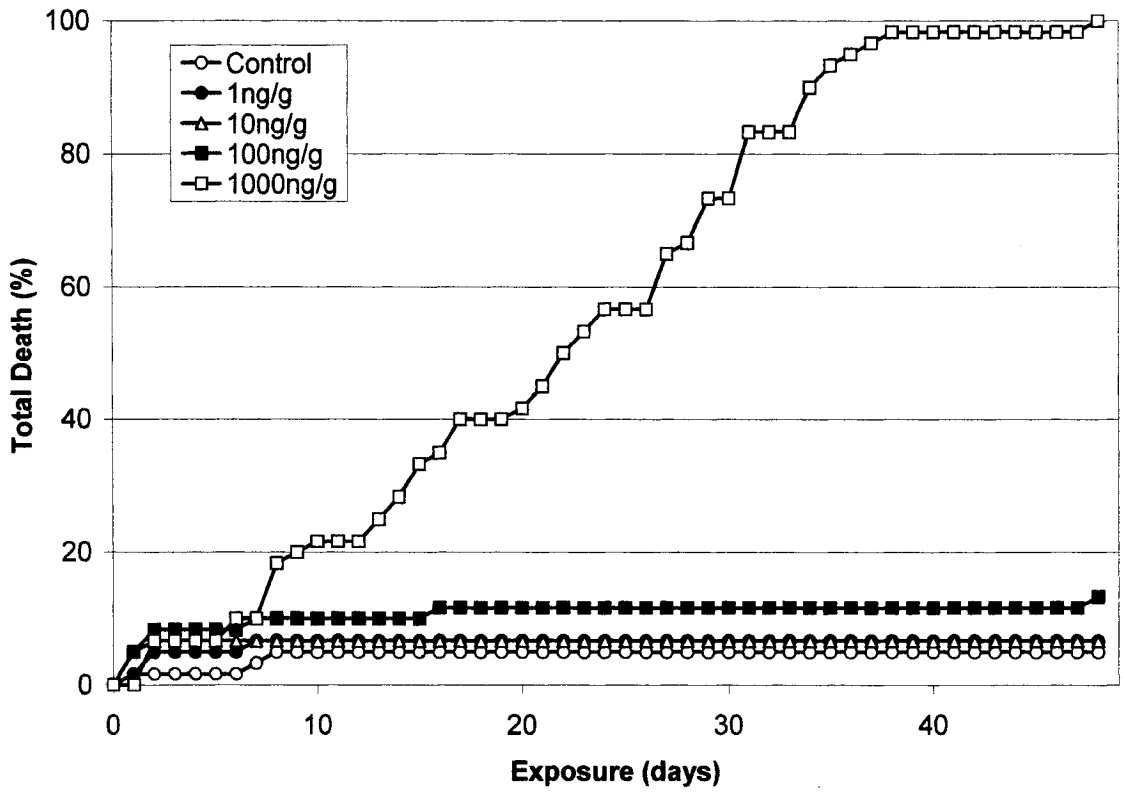

Figure 2.3 – Total cumulative mortality observed in the *R. pipiens* MeHg toxicity study, n=300.

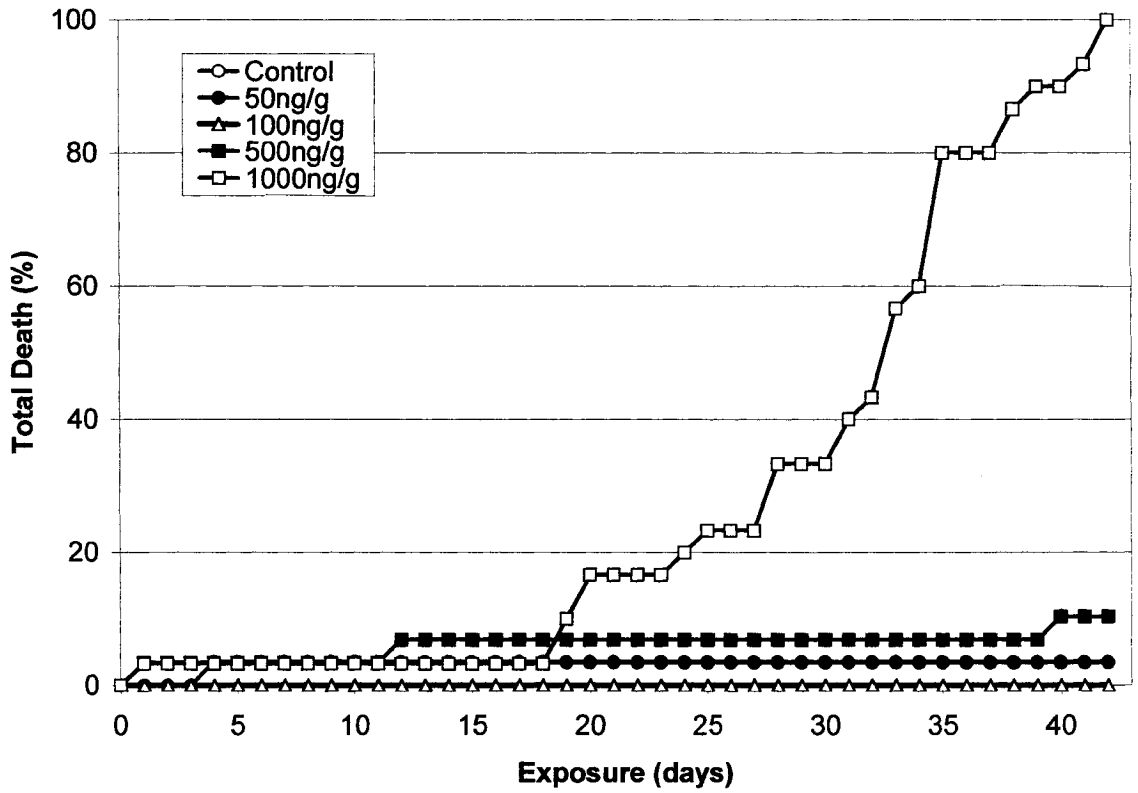

Figure 2.4 – Total cumulative mortality observed in the *X. tropicalis* MeHg toxicity study, n=146.

Table 2.2 – Subchronic LC50s calculated with day 33 data for all experiments using Trimmed Spearman Karber method. Significant difference (p=0.05) is estimated through examination of 95% confidence limits for overlap. Stage abbreviations are G – Gosner, NF – Nieuwkoop-Faber.

Species	**Stage**	**LC50 (ng/g)**	**95% Confidence Limit (ng/g)**	
			Lower	**Upper**
Bufo americanus	G25	323	301	347
Bufo americanus	G27-30	707[a]	391	1278
Rana pipiens	G25	371	306	450
Xenopus tropicalis	NF47	926[a,b]	743	1154

[a] - Significantly different (p=0.05) than *B. americanus* G25 LC50.
[b] - Significantly different (p=0.05) than *R. pipiens* G25 LC50.

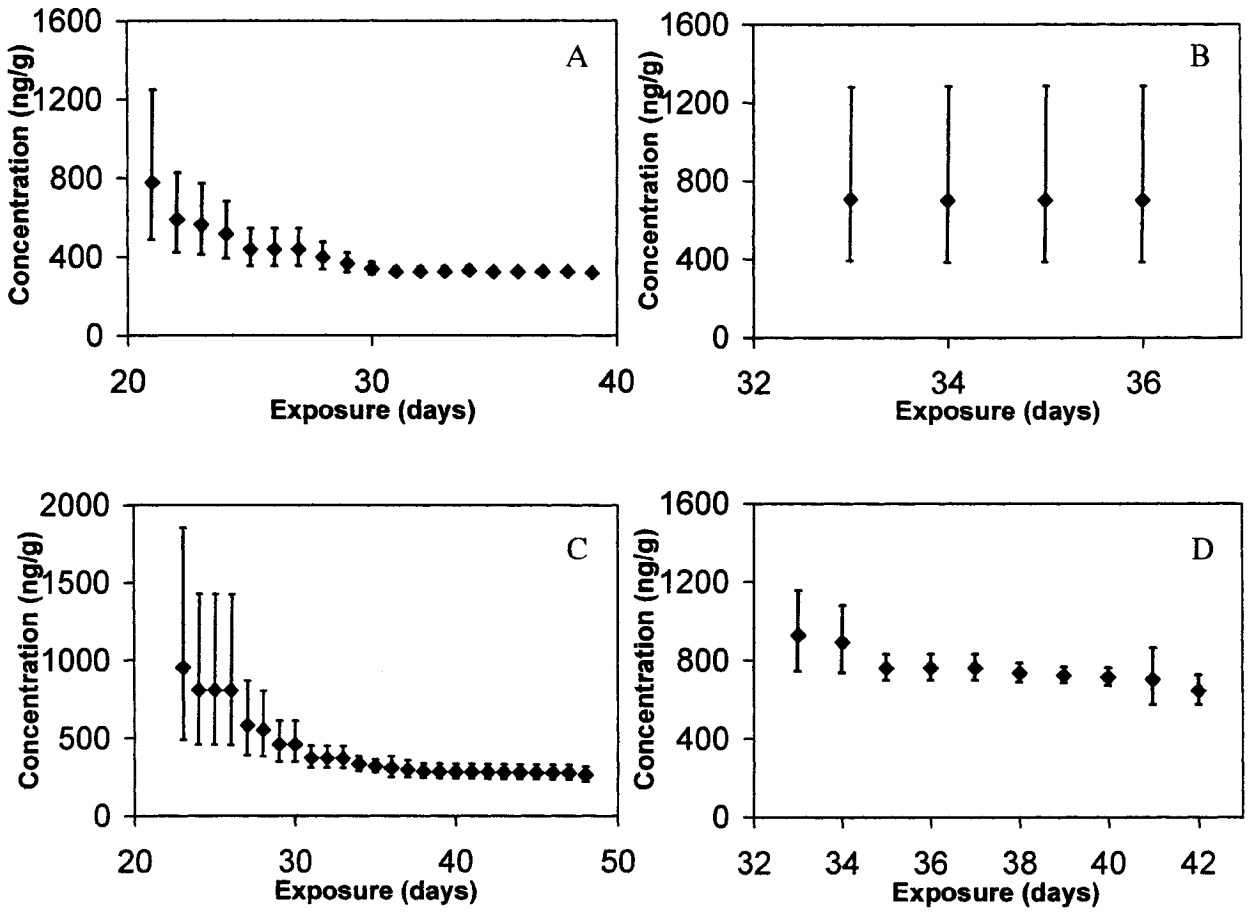

Figure 2.5 – LC50 estimate changes over the final days of the four MeHg toxicity studies, shown with 95% confidence limits. A) *B. americanus* G25 study, the final five points had incalculable 95% confidence limits. B) *B. americanus* G27 study only had four days where LC50s could be estimated, which only differed by 6ng/g. C) *R. pipiens* G25 study. D) *X. tropicalis* NF47 study, it is unclear why the 95% confidence limits increased at the end of this trial.

exposure period, LC50 estimates decreased in all species. The 95% confidence limits also undergo a reduction over the final days of the toxicity studies.

2.3.4.2 Developmental differences

Metamorphosis was not seen in all species as these experiments were short term and not meant to encompass the entire period of metamorphic development. Where metamorphosis did occur, juveniles were removed from the experiment.

2.3.4.3 Deformities and Behavioural Changes

Schematics of the pattern of onset for deformities and behavioural changes are presented for *B. americanus* (Figure 2.6), *R. pipiens* (Figure 2.7) and *X. tropicalis* (Figure 2.8). The final proportions of abnormalities are given in Table 2.3 for all species.

There were timing similarities between species in terms of specific symptom onset. Lethargy onset occurred only one day apart for the G25 and G27 experiments in *B. americanus* (day 15-16) whereas *R. pipiens* and *X. tropicalis* both developed lethargy on day 7. The onset of emaciation occurred within the same week in both *B. americanus* experiments (day 8 and 12). This was also seen in the *X. tropicalis* and *B. americanus* G27 experiments (day 12 and 17). *B. americanus* G27 and *X. tropicalis* developed kinks within the same week (day 8 and 13), whereas *B. americanus* G25 and *R. pipiens* developed kinks at day 21-22. All inversion observations occurred at a similar time (day 29-33). Lordosis and bloating occurred differently across all experiments. Additionally, one tadpole from the 1000ng/g treatment was observed to have "shudder swimming" behaviour in the

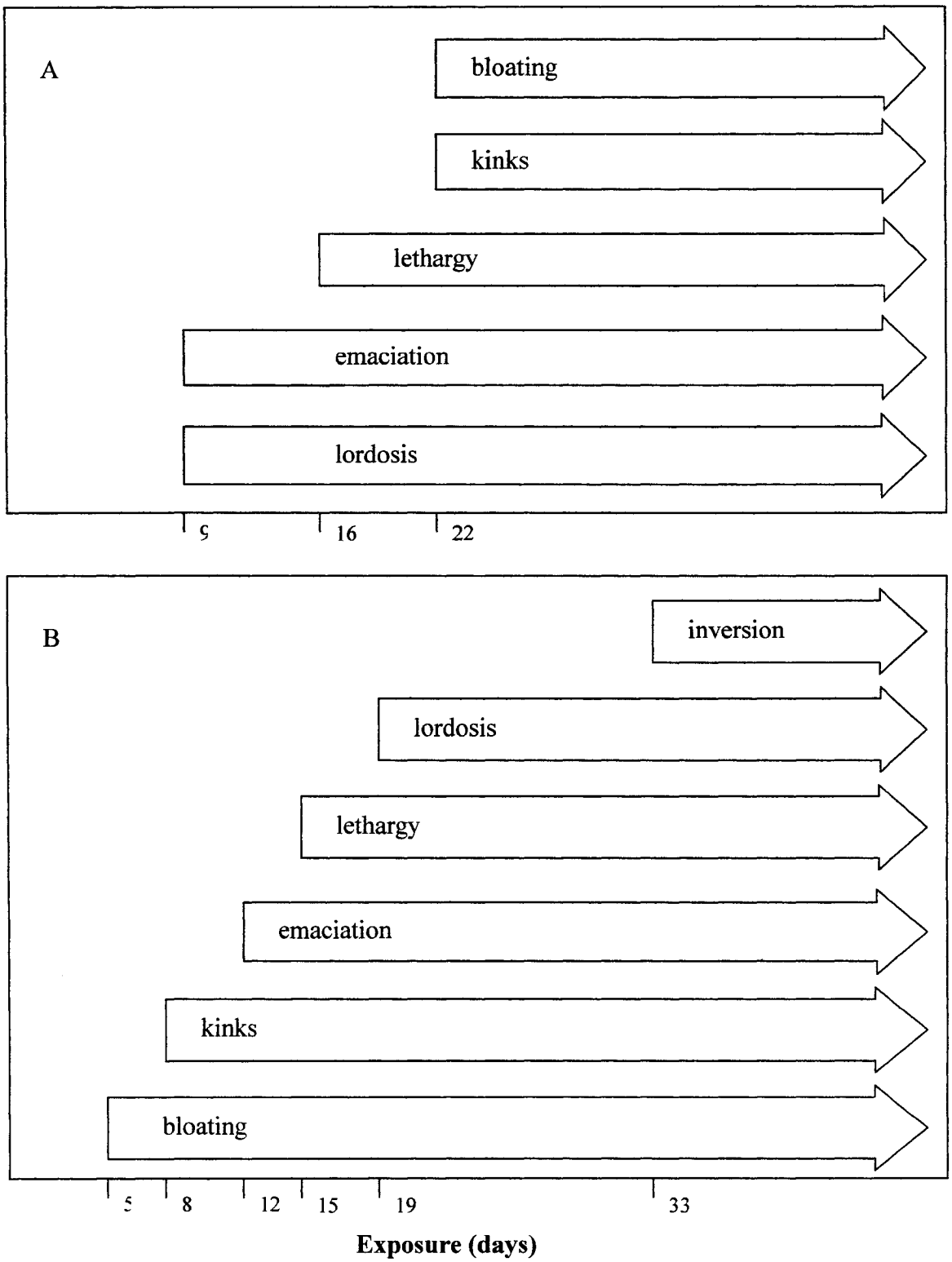

Figure 2.6 – Schematic representation of tadpole behavioural and physical abnormalities for the *B. americanus* MeHg toxicity studies. Arrows indicate that once an abnormality was noted within the experiment, it continued to be observed until experimentation ended. Day of onset is provided. A) *B. americanus* G25 study, n=298. B) *B. americanus* G27 study, n=192.

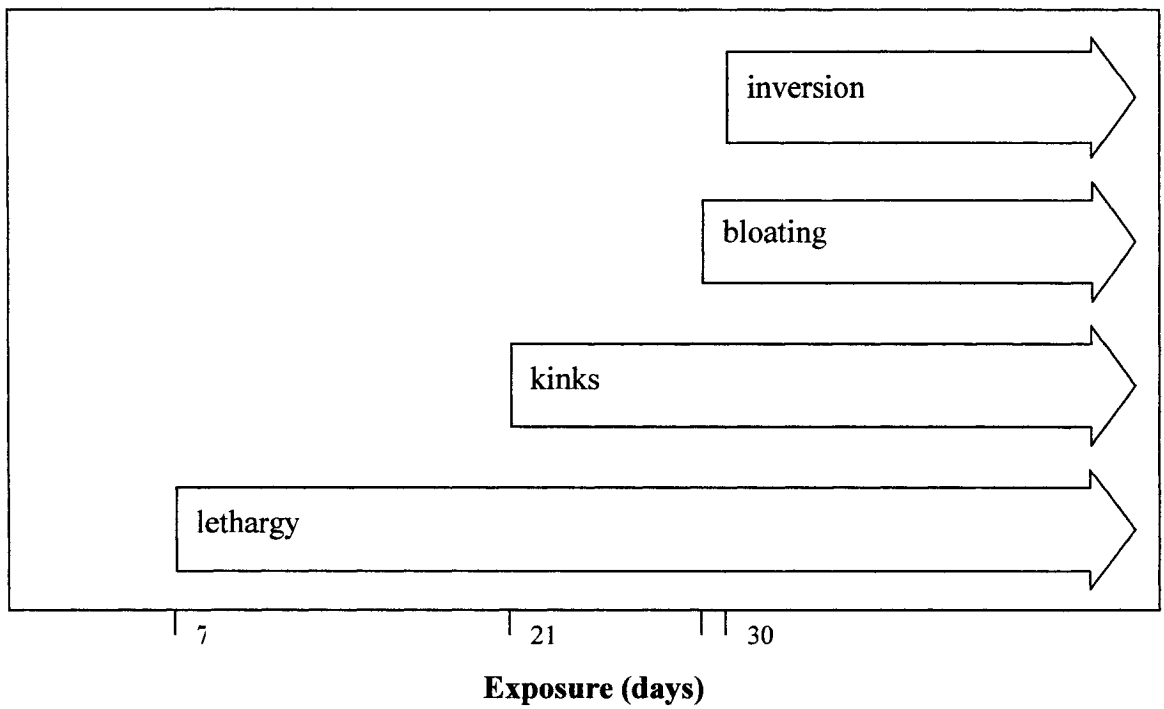

Figure 2.7 – Schematic of tadpole behavioural and physical abnormalities by day of onset for *R. pipiens* G25 MeHg toxicity study, n=300. Arrows indicate that once an abnormality was noted within the experiment, it continued to be observed until experimentation ended.

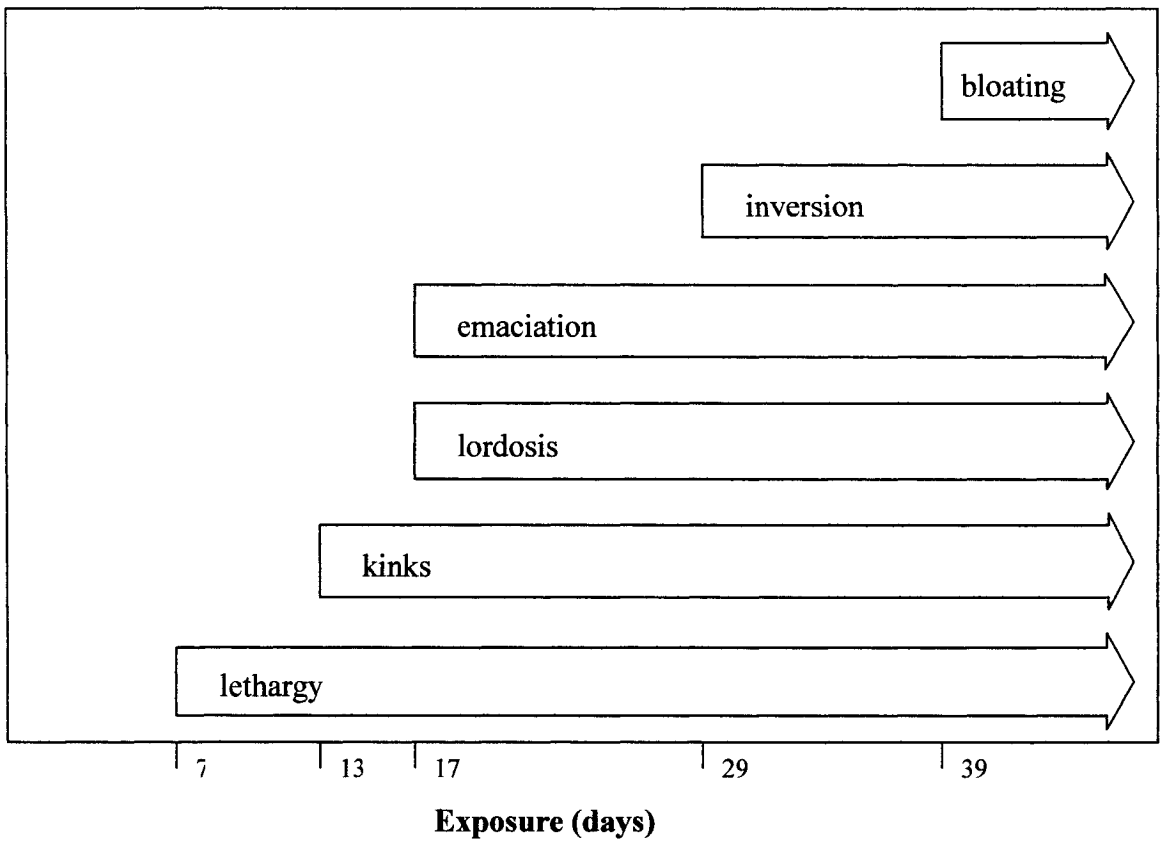

Figure 2.8 – Schematic representation of tadpole behavioural and physical abnormalities by day of onset for *X. tropicalis* MeHg toxicity study, n=146. Arrows indicate that once an abnormality was noted within experiment, it persisted until the experiment terminated.

Table 2.3 – Final proportions of deformities and behavioural changes noted in MeHg toxicity studies for all species. Proportions are shown with treatment levels in parentheses where the symptoms were identified.

Test	N	Abnormality					
		Lethargy	**Emaciation**	**Inversion**	**Kinks**	**Lordosis[a]**	**Abdominal[b]**
B. americanus G25	298	1.0 (1000)	0.07 (1) 0.02 (100) 0.03 (1000)	0.0	0.02 (10)	0.02 (C) 0.03 (1) 0.07 (1000)	0.05 (C) 0.03 (1) 0.02 (10) 0.12 (100) 0.02 (1000)
B. americanus G27	192	0.03 (10) 1.0 (1000)	0.05 (1) 0.21 (10) 0.11 (100) 0.28 (1000)	0.03 (100)	0.08 (10) 0.08 (1000)	0.03 (100) 0.03 (1000)	0.03 (C) 0.05 (1) 0.15 (10) 0.16 (100) 0.13 (1000)
R. pipiens G25	300	1.0 (1000)	0.0	0.03 (1000)	0.03 (1)	0.0	0.02 (1000)
X. tropicalis NF47	146	0.03 (500) 1.0 (1000)	0.07 (1000)	0.07 (500) 0.50 (1000)	0.76 (C) 0.64 (50) 0.60 (100) 0.28 (500) 0.03 (1000)	0.03 (500) 0.13 (1000)	0.03 (100)

[a] – Lordosis includes abnormalities also described as curved

[b] – Abdominal includes oedema, general swelling across the gut and localized outpouchings of gut, described as lumps

B. americanus G25 experiment on experimental day 28. Although no other tadpoles exhibited this problem in swimming, it was a distinctive abnormality.

2.3.4.4 Body burden

Quality control and quality assurance samples were run during the MeHg digestions. Blanks were below the detection limit (<0.02pg/g) and contributed no MeHg to samples. Average pooled standard reference material recovery was 120±6% of the expected value, which is slightly elevated but still acceptable. Ideally, recovery values would fall between 90 and 110%. Since the blanks contributed no MeHg to samples, it is unlikely this increased recovery is due to contamination. Average percent difference between replicates was 9.7±7.5%.

Body burden measurements in the *B. americanus* G25 experiment were available only for the final day cull in the control and intermediate treatment levels, as little death occurred over time for these levels. The best measure of body burden over time exists for the highest treatment level, 1000ng/g, as death occurred steadily throughout the experiment.

Average MeHg body burdens increased with an increase in treatment concentration (Figure 2.9). Control and 1ng/g treatments are approximately equal, with 1ng/g body burdens 4.7-fold higher than Controls. Body burdens in the 10ng/g treatment are eight-fold higher than 1ng/g, and the 100ng/g body burdens are 24-fold higher than the 10ng/g. Tadpoles in the 1000ng/g treatment died over the course of the experiment, thus a representation of body burden over time could be plotted. As time progressed, the concentration of MeHg measured in tadpoles increased (Figure 2.10). No points were below 4000ng/g.

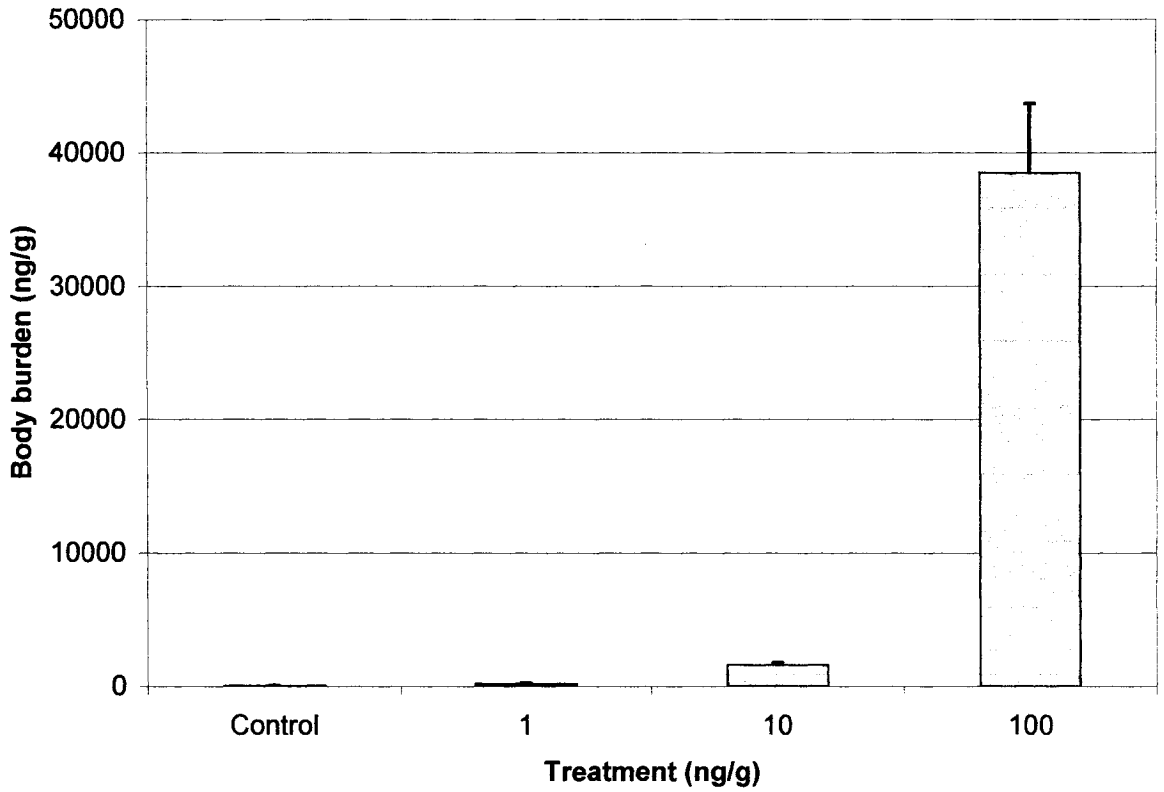

Figure 2.9 – Average body burden levels in the *B. americanus* G25 toxicity experiment on the final day, shown with standard error. Controls contained 42 ± 6ng/g, which is not clearly seen in this scale. $N_{Control} = 41$, $N_{1ng/g} = 40$, $N_{10ng/g} = 33$, $N_{100ng/g} = 39$.

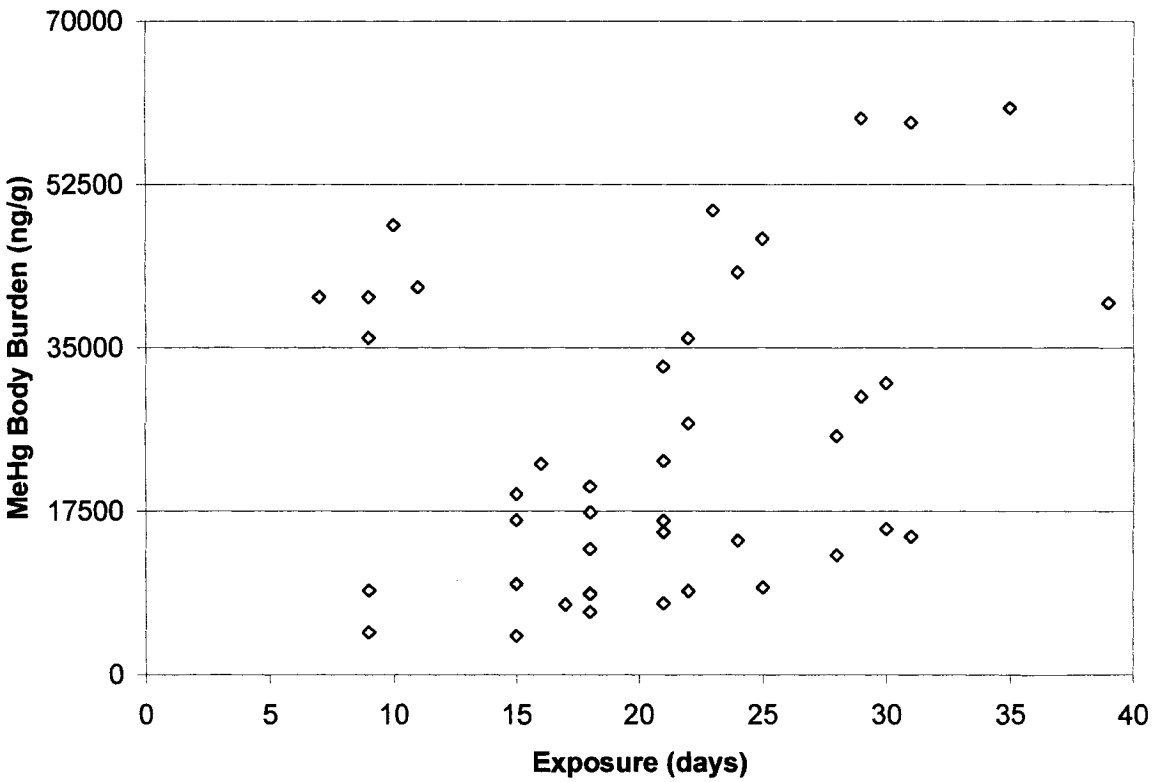

Figure 2.10 – MeHg body burden at death over time in the *B. americanus* G25 MeHg toxicity study 1000ng/g treatment. N=60.

In the *B. americanus* G27 exposure, the representation of body burden over time was facilitated by the gradual metamorphosis which occurred in all treatments, in addition to death over time in the highest treatment (Figure 2.11). To enable the presentation of all treatments within the same figure, these body burdens have been placed on a logarithmic y-axis scale. With little overlap, each treatment involves an increase of approximately one order of magnitude over the previous treatment. Body burdens were also graphed separately to show individual treatment trends more clearly. Within each treatment there is an increase in body burden as time progresses (Figures 2.12-2.16). In total, the body burdens measured in this study span six orders of magnitude, although the majority of points fall within the centre four orders of magnitude.

The *R. pipiens* G25 experiment had low mortality over time in the first four treatment levels. The average final day body burdens show the increase in MeHg accumulation as exposure level increased, with a large increase accompanying the 100ng/g treatment compared to the other treatments (Figure 2.17). The 1ng/g treatment is 2.5-fold higher than Controls. The 10ng/g average body burdens are five-fold higher than 1ng/g, whereas the 100ng/g body burdens are nine-fold higher than the 10ng/g. At the 1000ng/g treatment level, more MeHg was measured in tadpoles over time. Body burden concentrations are higher than those seen in the *B. americanus* G25 experiment (Figure 2.18).

The *X. tropicalis* NF47 experiment had low mortality in the lower level treatments. The trend of increased body burden with increased experimental level was also observed on the final day in this experiment (Figure 2.19). A 74-fold increase in body burden occurred between the Control and 50ng/g treatment levels. Between the 50ng/g and 100ng/g

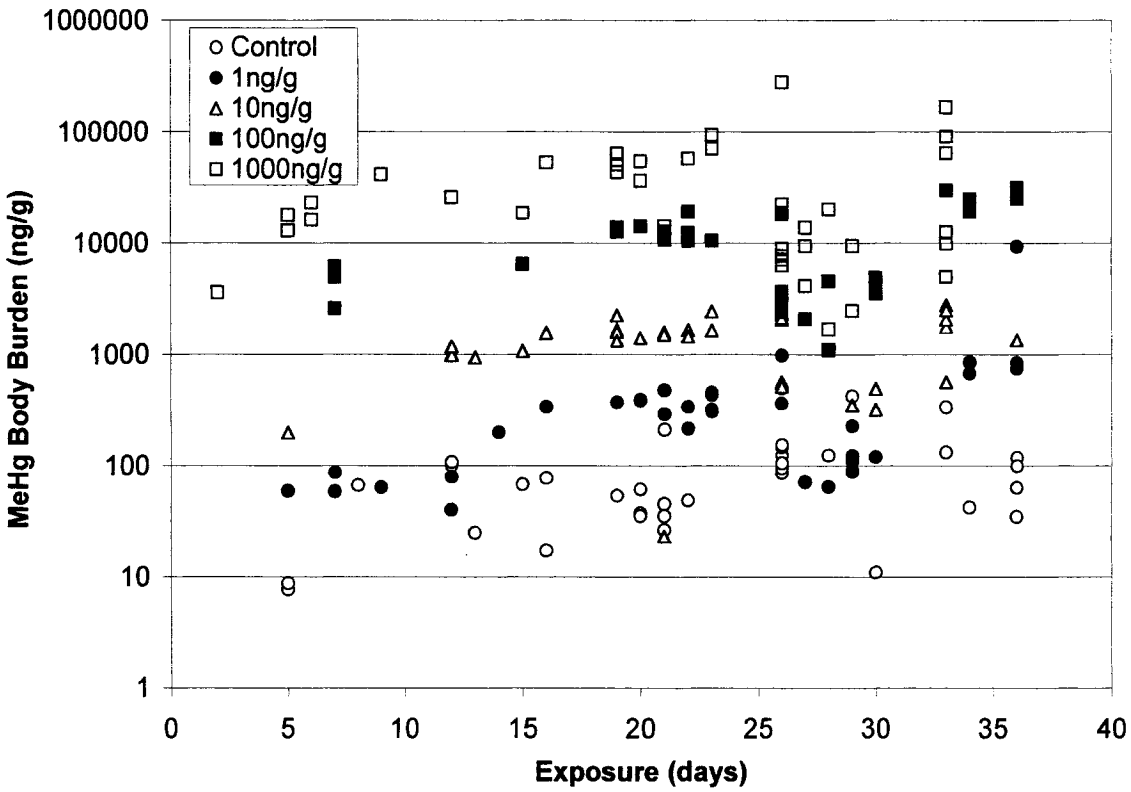

Figure 2.11 – Body burden over time at death or metamorphosis for the *B. americanus* G27 toxicity study for all treatment levels. Body burden data are graphed on a logarithmic y-axis to show the separation of each of the treatment levels. Body burden over time was shown for all treatments since tadpoles were regularly removed from the experiment, either through metamorphosis which occurred in all treatments or the gradual death which occurred in the highest treatment level, 1000ng/g. $N_{Control} = 37$, $N_{1ng/g} = 38$, $N_{10ng/g} = 36$, $N_{100ng/g} = 37$, $N_{1000ng/g} = 39$.

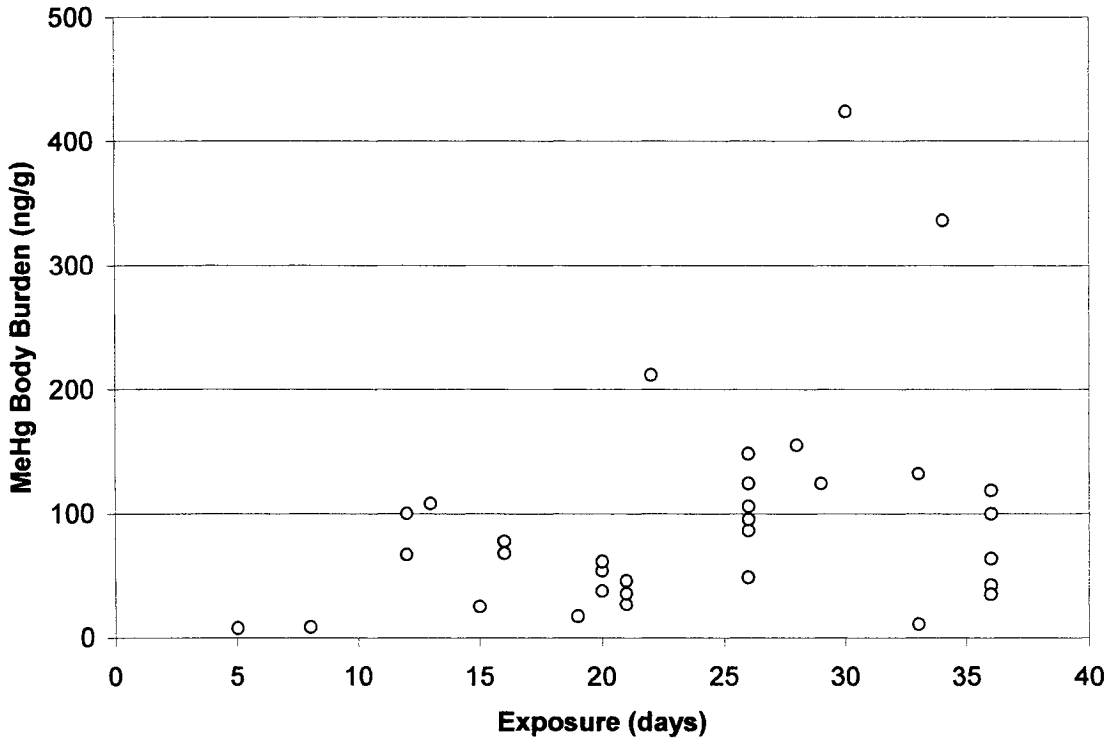

Figure 2.12 – MeHg body burden after death or metamorphosis in the Control treatment of the *B. americanus* G27 toxicity study. N=37.

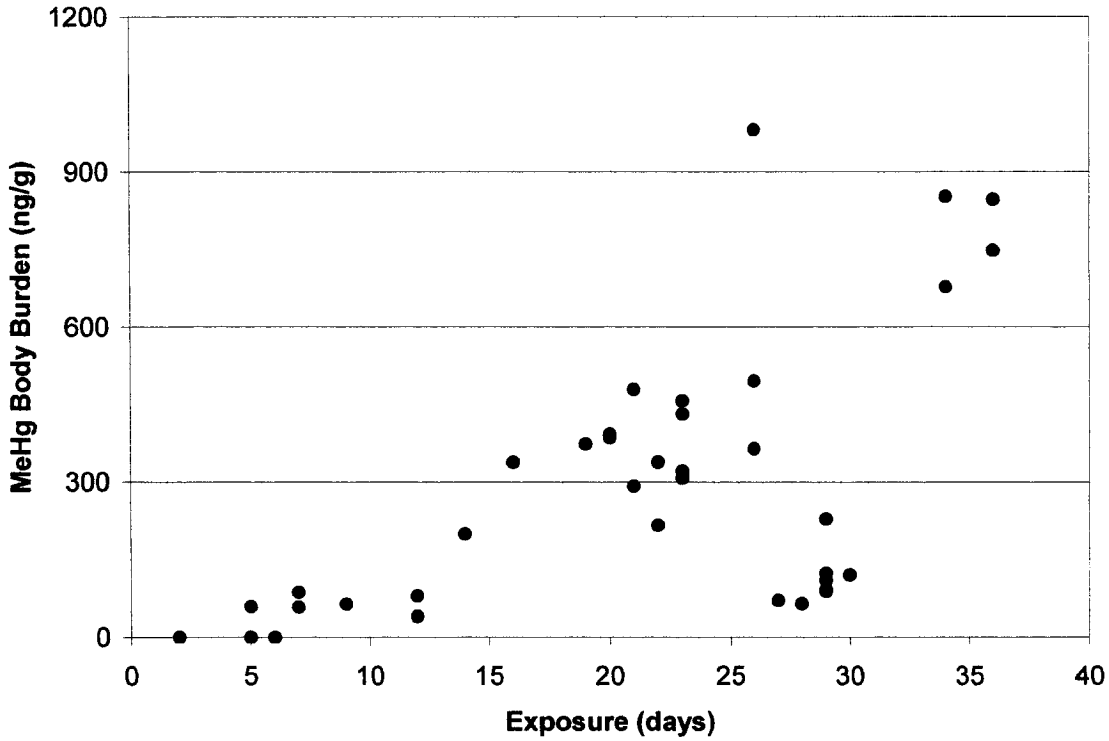

Figure 2.13 – MeHg body burden after death or metamorphosis in the 1ng/g treatment of the *B. americanus* G27 toxicity study. N=38.

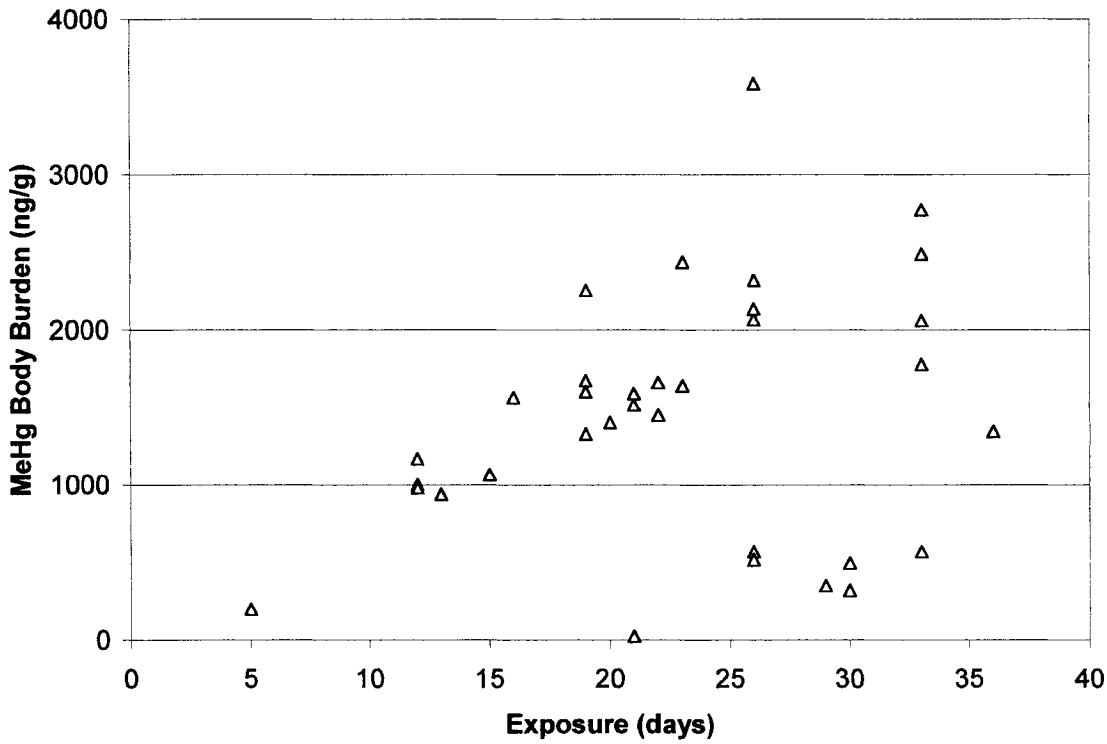

Figure 2.14 – MeHg body burden at death or metamorphosis for the 10ng/g treatment of the *B. americanus* G27 toxicity study. N=36.

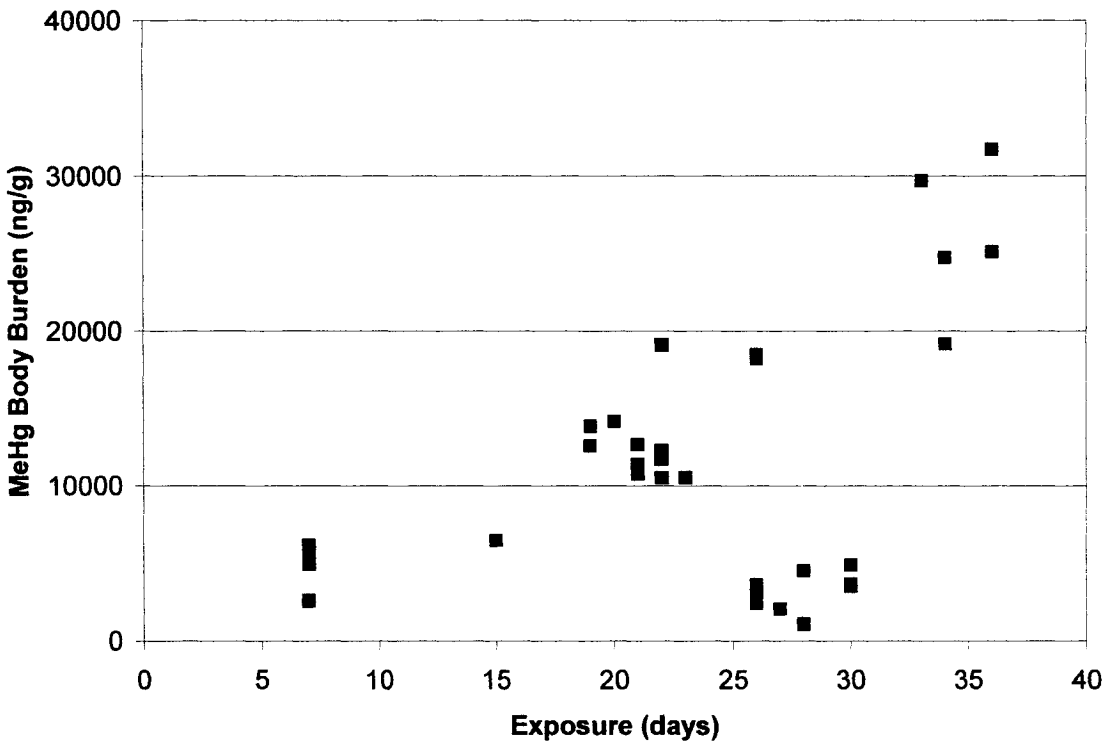

Figure 2.15 – MeHg body burden at death or metamorphosis in the 100ng/g treatment of the *B. americanus* G27 toxicity study. N=37.

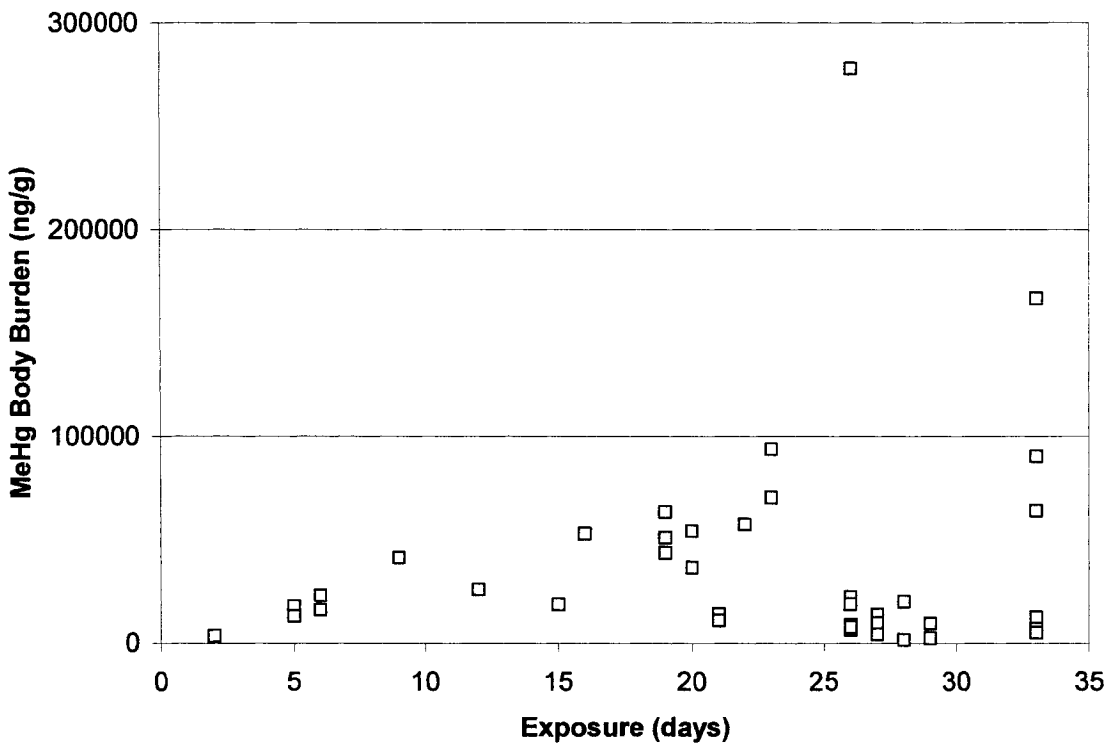

Figure 2.16 – MeHg body burden at death or metamorphosis in the 1000ng/g treatment of the *B. americanus* G27 toxicity study. N=39.

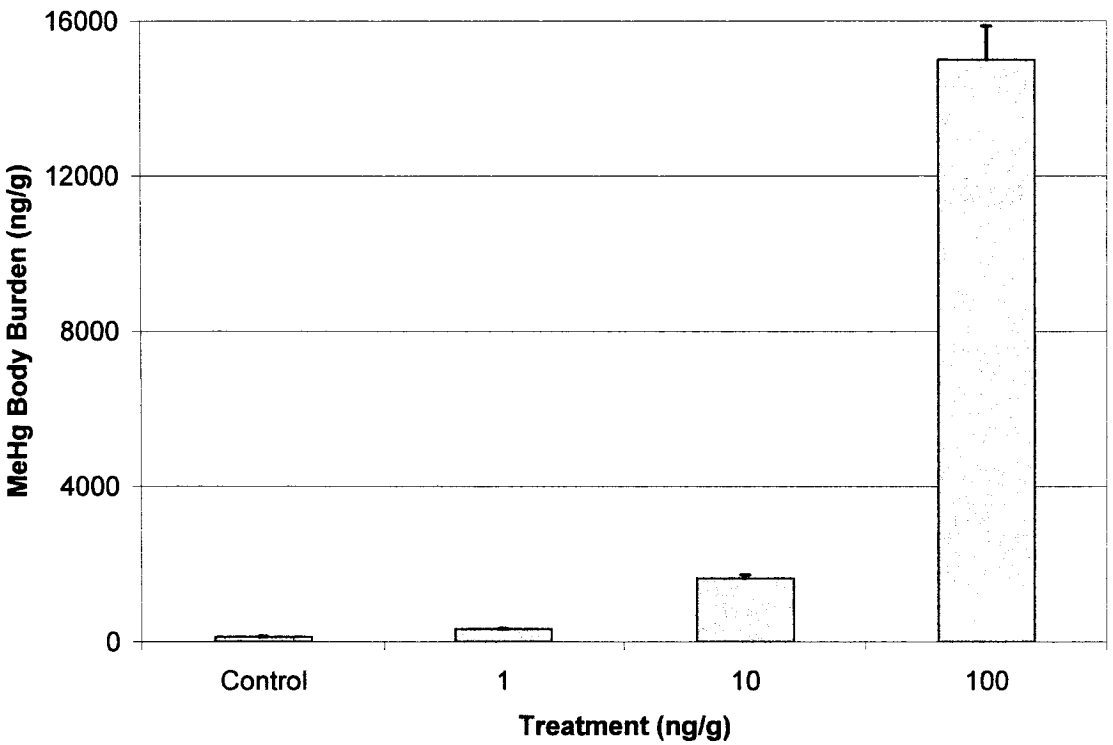

Figure 2.17 – Average MeHg body burden at death in the lower treatment levels of the *R. pipiens* G25 toxicity study, shown with standard error. Averages are taken for the final day cull body burden. $N_{Control} = 29$, $N_{1ng/g} = 36$, $N_{10ng/g} = 42$, $N_{100ng/g} = 53$.

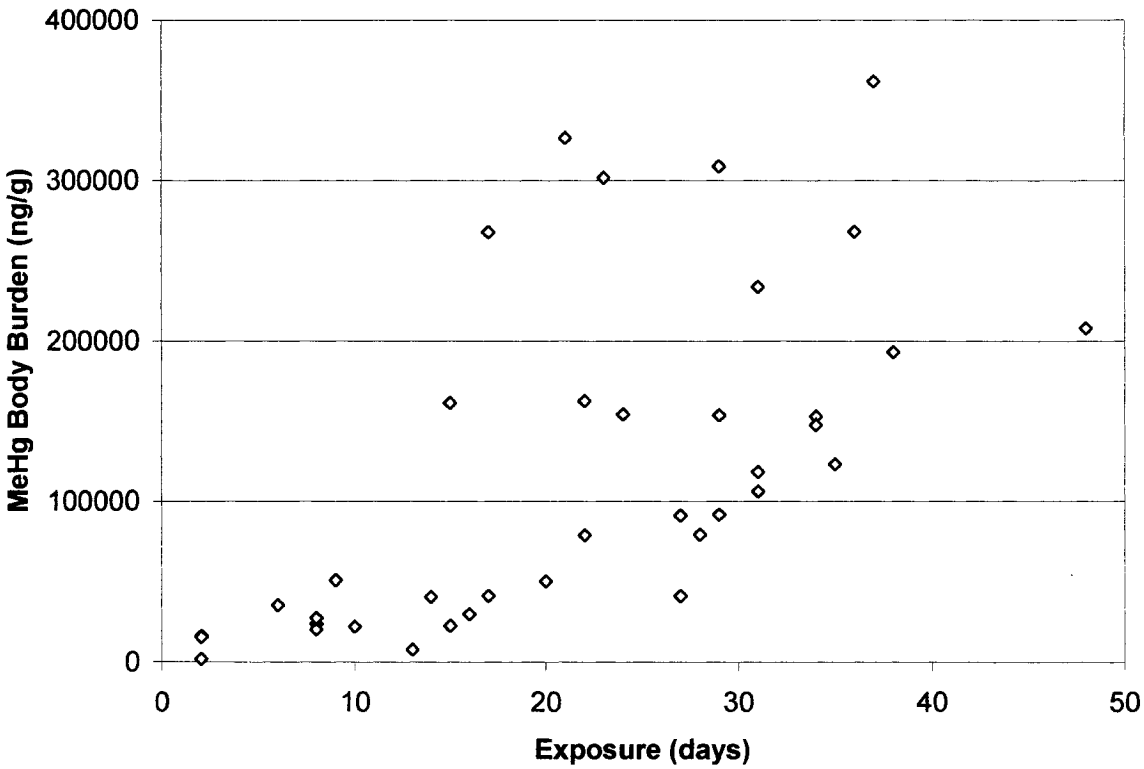

Figure 2.18 – MeHg body burden at death over time in the *R. pipiens* G25 toxicity study 1000 ng/g treatment level. N=60.

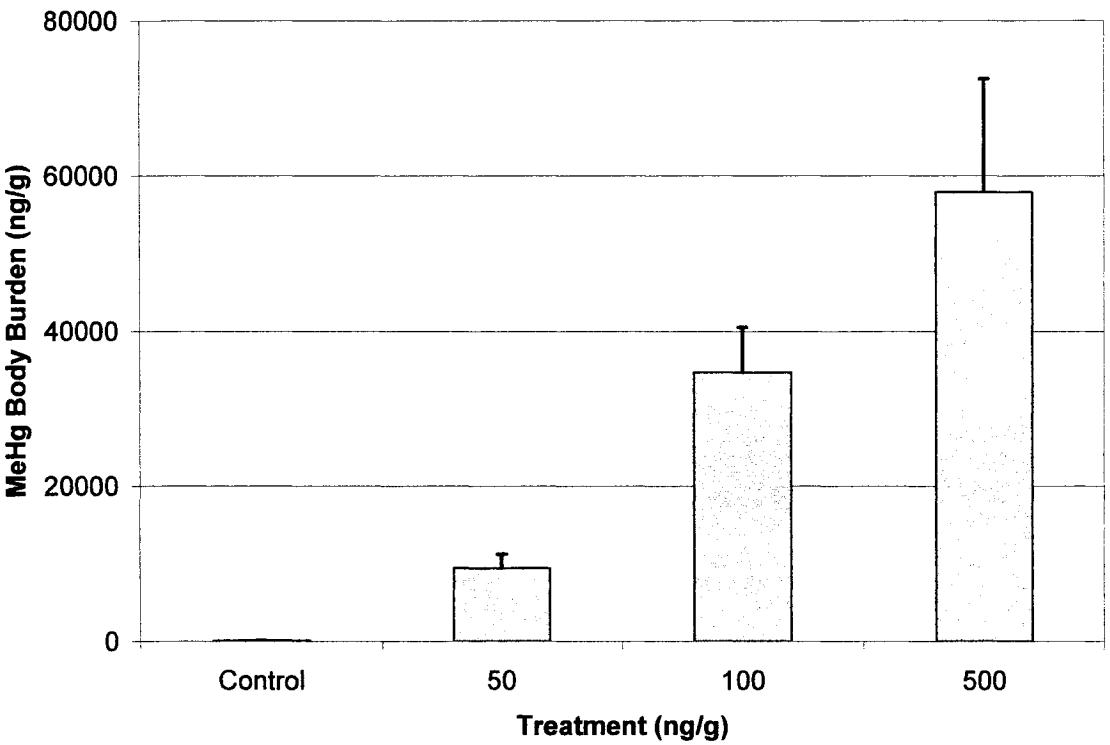

Figure 2.19 – Average MeHg body burden for lower treatment levels in the *X. tropicalis* NF47 toxicity study, shown with standard error. All averages are calculated with final day body burden numbers. $N_{Control} = 20$, $N_{50ng/g} = 22$, $N_{100ng/g} = 25$, $N_{500ng/g} = 18$.

treatment, there was a 3.7-fold increase in body burden, and a 1.7-fold increase in body burden between the 100ng/g and the 500ng/g treatment. The *X. tropicalis* 1000ng/g treatment had a slight decline in body burden over the final 24 days of experimentation (Figure 2.20), which is different than any other experiment.

Environmental samples taken during field sampling had MeHg body burden concentrations ranging from 1ng/g to 85ng/g (Figure 2.21). The higher level environmental samples, such as the tadpoles from the Chelsea man made sand quarry, are similar to the concentrations measured in Controls of the *B. americanus* G25 experiment. All other experimental treatments in the four studies have body burdens where the lowest concentration is much higher than the highest environmental level. Detritus and periphyton environmental samples are presented in Table 2.4, and range from 3.1 to 85.0ng/g and 0.27 to 10.9ng/g, respectively. Water samples taken during field sampling had MeHg concentrations ranging from 0.38 to 0.77ng/L.

2.4 Discussion

This is one of the first studies to examine the effects of dietary MeHg on anuran tadpoles. Our results show that orally administered MeHg is toxic to the tadpoles of 3 anuran species. Mortality rarely occurred in low level treatments but the highest treatment level elicited a death response within a week of application for the two native species Gosner stage 25 toxicity studies (Figures 2.2 and 2.3). The *B. americanus* G27 toxicity study indicated a greater resistance to MeHg, as a long lag period occurred before mortality increased. *X. tropicalis* was less sensitive to MeHg toxicity, as most mortality did not occur until after day 18 in this study (Figure 2.4). *X. tropicalis* responded very differently to

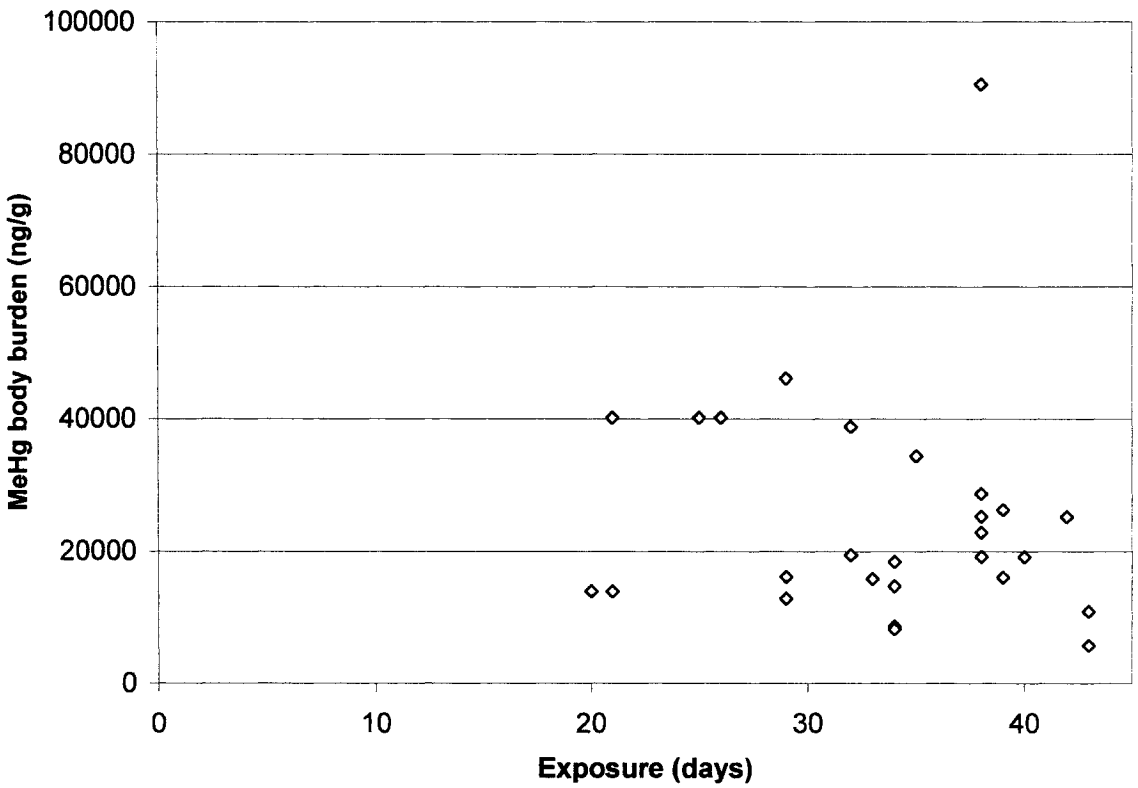

Figure 2.20 – MeHg body burden at death over time for the *X. tropicalis* NF47 toxicity study 1000ng/g treatment level. N=30.

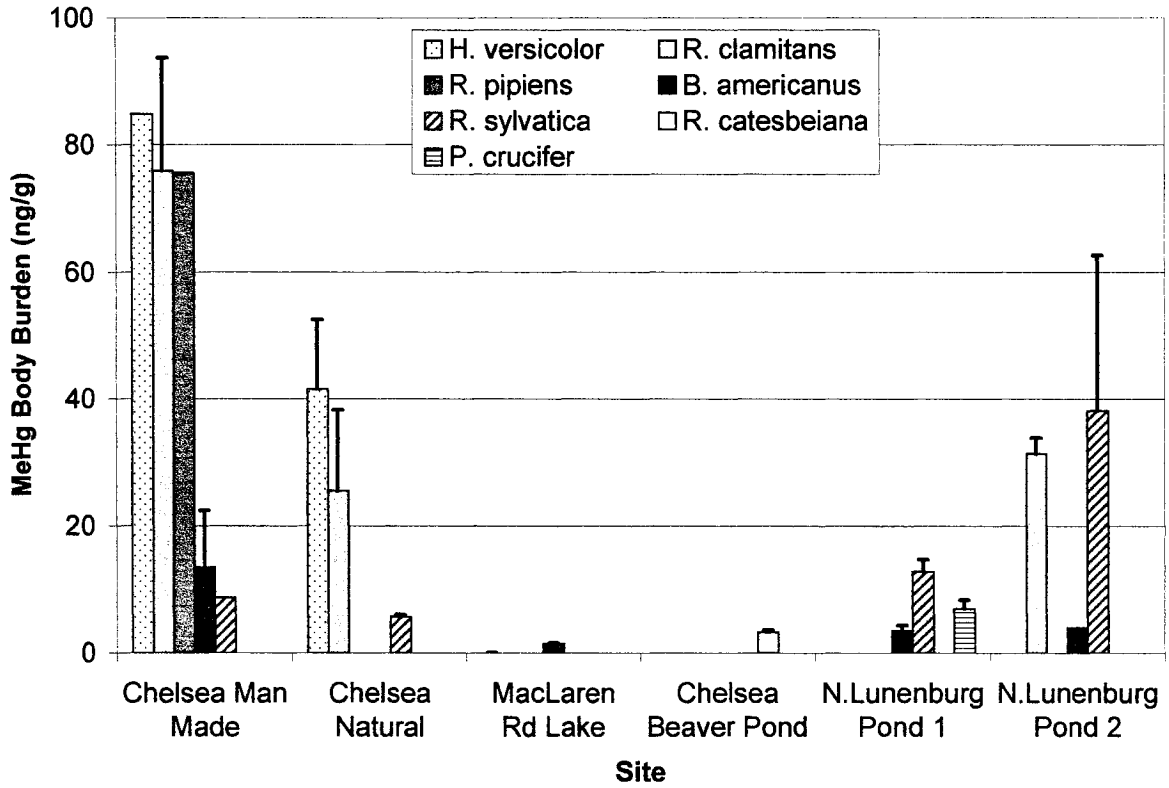

Figure 2.21 – Average MeHg body burden found in tadpoles gathered from unpolluted lakes and ponds in Eastern Ontario and Western Quebec. Standard error is presented for samples with n>1 average body burden values. Certain points are representative of one pooled sample or one individual, thus no standard error was calculable. Sample numbers are as follows for samples n>1 : Chelsea Man Made - $N_{R.clamitans} = 5$, $N_{B.americanus} = 3$; Chelsea Natural - $N_{H.versicolor} = 4$, $N_{R.clamitans} = 4$, $N_{R.sylvatica} = 2$; MacLaren Rd. Lake - $N_{B.americanus} = 47$; Chelsea Beaver Pond - $N_{R.catesbeiana} = 5$; N.Lunenburg Pond 1 - $N_{B.americanus} = 61$, $N_{R.sylvatica} = 10$, $N_{P.crucifer} = 17$; N.Lunenburg Pond 2 - $N_{R.clamitans} = 9$, $N_{R.sylvatica} = 7$.

Table 2.4 – Average environmental MeHg concentrations in samples gathered from Eastern Ontario and Western Quebec lakes and ponds. Dashed lines indicate that the sample was not collected or found at that location. Standard deviations are provided for samples n>1.

	(ng/L)	Sample (ng/g)		
Location	**Water[g]**	**Periphyton[a]**	**Detritus[b]**	**Insects[c]**
Chelsea Man Made	0.56 ± 0.07	0.27 ± 0.78[e]	2.56 ± 1.89[f]	14.07
Chelsea Natural	0.59 ± 0.09	3.82 ± 2.88[g]	4.43	118.21
MacLaren Rd Lake	0.68 ± 0.03	4.86	5.64	-----
N. Lunenburg Pond 1	0.77 ± 0.21	10.91	7.43	140.96
N. Lunenburg Pond 2	0.38 ± 0.22	-----	85.05[d]	-----

a – Periphyton and algae attached to substrate
b – Detritus includes suspended particulate matter
c – Insects measurement made from a sample of various insects collected from ponds, including caddisflies, spider mites, damselfly larvae and snails
d – Detritus from N. Lunenburg Pond 2 contained large amounts of zooplankton which could not be separated from the sample
e – N = 4
f – N = 3
g – N = 2

MeHg ingestion compared to *B. americanus* and *R. pipiens* G25 experiments, and seems to be as resistant as the tadpoles in the *B. americanus* G27 experiment. This could be due to the stage equivalency of NF47 in *X. tropicalis* to G25 through to G27 for *R. pipiens* and *B. americanus* (Table 2.1).

Estimates of LC50 confirm a difference in sensitivity between the species, as well as between developmental stages. *B. americanus* G25 tadpoles show a significantly higher sensitivity to MeHg than *B. americanus* G27 tadpoles (Table 2.2). Therefore, an early stage tadpole ingesting high MeHg will have decreased chances of survival. However, later stage tadpoles also experience toxic effects from high MeHg (Table 2.3). The *R. pipiens* and *B. americanus* G25 experiment LC50 values were not significantly different, thus these species are similarly sensitive to MeHg.

Although MeHg uptake is more likely to be through the diet (Hall et al. 1997), no previous LC50 studies involving amphibian MeHg ingestion were identified in extensive literature searches. Previous LC50s have been performed using water-borne MeHg exposure to concentrations 10^3 to 10^6 times those seen in nature. Some water exposure LC50 estimates are 60μg/L MeHgCl for *R. breviceps* tadpoles and 56μg/L MeHgCl for *B. melanostictus* tadpoles over 96 hours (Linder and Grillitsch 2000). Chang et al. (1974) showed *R. pipiens* experienced 100% mortality at 50μg/L MeHgCl within 48 hours, and those exposed to 1 to 10μg/L MeHgCl experienced arrested metamorphic development until the study was terminated. Compared to these concentrations, our results seem to show a relative insensitivity to MeHg, with LC50 estimates ranging from 323ng/g MeHg to 926ng/g MeHg. Yet, natural water concentrations are not as high as those found in laboratory studies and natural food sources for tadpoles can reach the concentrations used in this study (Figure

2.1). These higher concentrations that tadpoles are exposed to through diet are the reason that this is the environmentally relevant avenue of research.

The chemical and biological processes involved in the digestion of MeHg can have a great impact on toxicity. MeHg binds preferentially to sulfhydryl functional groups, found abundantly in proteins and amino acids like glutathione and cysteine. It has been shown that MeHg is reabsorbed from the intestine and the biliary tree, even after it has been bound by glutathione and released into the intestine for excretion (Clarkson 1994). MeHg is capable of passing through the blood-brain barrier when attached to cysteine, mimicking the amino acid methionine in the body (Simmons-Wallis et al. 2002). It is also possible that the damage inflicted on tadpoles by MeHg exposure in water is quite different from that through oral ingestion. Water exposure to inorganic Hg has caused damage to the gill structures of fish (Oliveira Ribeiro et al. 2002), and Chang et al. (1974) observed breathing difficulties in their exposure of *R. pipiens* tadpoles to concentrations between 50μg/L and 1000μg/L MeHgCl in water. It is possible that mortality in previous LC50 studies (Linder and Grillitsch 2000) is due to gill structure damage by the high concentrations of MeHg in the water causing suffocation rather than inducing toxicity to other body tissues.

Our LC50 estimates changed as time progressed, and if the studies were allowed to continue past 100% mortality in the highest treatment, the estimates could have been reduced further as the second highest treatment level tadpoles died. For example, by the end of the *X. tropicalis* NF47 study, the 500ng/g treatment level was beginning to show the same symptoms of MeHg poisoning as the 1000ng/g treatment tadpoles did at the outset of experimentation (Table 2.3), and the LC50 estimate was reduced from 926ng/g to 643ng/g (Figure 2.5). This could indicate that LC50s are unsuitable estimates of MeHg toxicity. Due

to the mode of action of MeHg, bioaccumulating in tissues and affecting cellular functions and brain architecture, perhaps a tolerable intake estimate should be made, as it has been done for humans (Clarkson 1998) as it would be more applicable in investigating natural populations. The human tolerable weekly intake was derived by applying a "safety factor" of 10 to an estimate of the lowest effect level for Hg from a study in Japan (Clarkson 1998). Although we do not have the lowest effect level, using a conservative estimate of 1% of the LC50 value, a tolerable intake estimate for *B. americanus* G25 tadpoles is 3ng/g. Comparing this value with the MeHg available in the environment (Figure 2.1), we can see that a daily intake of 3ng/g of MeHg is possible for native amphibian species grazing on periphyton.

The timing of the appearance of some of the abnormalities suggests a common response across species and a common etiology, i.e. MeHg exposure. These abnormalities, such as emaciation and lethargy, can be directly related to past research into MeHg poisoning effects on animal physiology. Inversion, or the loss of righting reflex, can also be associated with the degeneration of the motor functions and damage to the cerebellum, as can the instance of "shudder swimming" behaviour seen in the *B. americanus* G25 experiment (Aulerich et al. 1974, Wren et al. 1987, Clarkson 1994, Wolfe et al. 1998). Other abnormalities, such as spinal kinking, are not as clearly linked to MeHg exposure, and may require further research to discover the cause. This is especially true in the case of *X. tropicalis*, where kinking seemed to exhibit an inverse relationship to MeHg concentration. The lowest levels of tail kinking were observed in the 1000ng/g treatment, at 3%, whereas in the 50ng/g treatment 64% of tadpoles had kinked tails. In the Control treatment 76% of tadpoles had kinking, which suggests that kinking may not be related to MeHg exposure, and

higher MeHg concentrations could have been inhibiting or interacting with another process which was causing the kinking. Lordosis, another spinal abnormality, did not exhibit this inverse relationship, and generally had an increasing or static trend with increasing MeHg concentration (Table 2.3), although proportions of this abnormality were also low in the affected treatment populations. Abdominal abnormalities did not follow any trend to suggest that they were related to MeHg exposure. Certain abnormalities observed in our study have also been observed previously in experiments conducted on animals higher in aquatic food chains and are linked with MeHg ingestion. Although commonly seen in other experiments involving tadpole exposure to alkylphenol-polyethoxylate breakdown products such as octylphenol (Crump 2000, Croteau unpubl.), abnormalities such as spinal curvature and oedema do not follow a trend with MeHg exposure and the appearance of these abnormalities may be incidental in this study.

Tadpole body burdens of MeHg increased over time through the exposure period and differed for each species. *B. americanus* body burdens increased slowly (Figures 2.10 and 2.11) while *R. pipiens* body burdens increased more rapidly (Figure 2.18). This increase in body burden over time was expected as previous research indicates that MeHg is very inefficiently removed from the bodies of higher organisms (Clarkson, 1994). The relative brevity of the exposure periods (36-48 days) also led to the expectation that there could not be enough time for MeHg release from the body, especially since tadpoles were constantly being exposed to MeHg in their daily food. However, *X. tropicalis* showed a static or potentially decreasing trend over the final 24 days of experimentation (Figure 2.20). Due to the lack of mortality or metamorphosis, no trend in body burden is known for the initial 20 days of exposure. Possible reductions in body burden late in the experiment could be due to

a loss of eating drive as MeHg poisoning occurs (Aulerich et al. 1974, Wren et al. 1987). MeHg could also be gradually lost during this time as tissues are resorbed and restructured during metamorphosis. The appearance of lethargy early in the experiment (Figure 2.8), especially in the highest treatment level, indicates that the tadpoles may be experiencing potential MeHg poisoning symptoms (Aulerich et al. 1974, Wren et al. 1987). The onset of emaciation by day 17 in *X. tropicalis* also suggests a possible reduction in feeding behaviour, but this was only seen in 7% of tadpoles in the 1000ng/g level (Table 2.3), and this decrease in body burden was not expressly evident in the *B. americanus* studies where proportions of emaciation were much higher. Further research into the relationship between accumulated MeHg body burden and effects on physiology should be undertaken.

In contrast to body burdens accumulated during experimentation, environmental levels were found to be low. Environmental body burdens in various species of tadpoles were only similar to the Controls of *B. americanus* G25 experiment. Tadpole samples were collected one to two months after egg deposition, so tadpoles had enough time to accumulate environmental MeHg. Periphyton and detritus had lower levels of MeHg (Table 2.4) than those in the literature for seston/phytoplankton and suspended particulate matter (Figure 2.1). This is a positive result when considering amphibian species developing within these local pristine environments, but research needs to be done into the environmental impacts of increasing pollution, such as that seen with mining activities, which mobilize more Hg into aquatic systems and food chains.

In conclusion, MeHg toxicity to amphibian tadpoles can be induced by oral ingestion. This is a more environmentally relevant study design as water MeHg levels are usually low in nature compared to the ability of MeHg to biomagnify within the food chain.

Early stage *B. americanus* and *R. pipiens* show similar sensitivity to ingested MeHg, whereas *X. tropicalis* and late stage *B. americanus* show significantly more resistance to MeHg as measured through LC50 estimates. Abnormalities commonly associated with MeHg poisoning such as lethargy, inversion and emaciation showed stronger trends than those associated with spinal curvature and oedema. Body burdens in native amphibian species showed increased MeHg over time however *X. tropicalis* exhibited a static or decreasing trend in MeHg body burden over the final 24 days of experimentation. This could be due to a reduction in feeding behaviour and lethargy, but further investigation is required to fully explain this phenomenon. Environmental concentrations and tadpole body burdens were found to be far lower than those measured for experimental tadpoles, indicating that the areas where field sampling occurred were pristine and tadpoles may not experience the negative effects of high level MeHg ingestion.

Chapter 3

3.0 Chronic methylmercury exposure effects on *Xenopus tropicalis* metamorphosis

3.1 Introduction

At chronic, low level exposure, MeHg could have effects on hormonal activity, cellular functions and gene transcription. Recent studies suggest that MeHg could act as a xenoestrogen as it is capable of binding and activating estrogen receptor α when tested *in vitro* in a breast cancer cell line (Martin et al. 2003). MeHg also disrupts microtubules (Hunter and Brown 2000) and therefore can affect processes dependent on microtubules such as neuronal migration and cell division. It has been shown that exposure to MeHg results in DNA damage (Gauthier et al. 1998, Taddei et al. 2001). The present research is based on the hypothesis that MeHg could potentially act as an environmental stressor, activating the hypothalamic-pituitary-interrenal stress axis in developing tadpoles to change the rate of metamorphosis. It is important to investigate the chronic exposure effects of low level MeHg ingestion on amphibian tadpoles. For this study, chronic was defined as a significant portion of a tadpole's developmental period with daily exposure to the chemical of interest and no significant death occurring during the first seven days of experimentation.

Aquatic organisms, such as fish, are affected by MeHg, resulting in the same effects discovered in piscivorous wildlife studies, such as liver, kidney and brain damage (Oliveira Ribeiro et al. 2002, Berntssen et al. 2003). As was discussed previously (Chapter 2), amphibian larvae are aquatic organisms existing in wetlands which are areas of MeHg production. Additionally, amphibians have not been studied for MeHg ingestion effects. We found tadpoles displayed symptoms of MeHg poisoning when ingesting elevated environmental concentrations. Unrine et al. (2004) showed an increase in the rate of

metamorphosis as measured through a decrease in days post-hatch to hindlimb and forelimb emergence in *R. sphenocephala* tadpoles fed periphyton grown in Hg-enriched mesocosms. Periphyton MeHg levels were measured and found to be elevated above their control diet, however their enrichment method used inorganic Hg instead of MeHg.

In nature, amphibian survival through metamorphosis is very low due to predation, loss of habitat and competition (Berven 1990). Any reduction in a tadpole's ability to evade predation or feed, through lethargy or loss of motor control, will result in a higher risk of mortality and a lower success rate. The developmental plasticity of amphibians through response to environmental stresses is important to allow tadpoles the ability to develop appropriately in changing conditions, for example the desert dwelling western spadefoot toad (Denver 1997). If the rate of development is slowed through other stressors, adulthood may not be reached before loss of habitat through natural seasonal pond drying occurs (Denver 1997). But if the rate of development is increased through stress at an earlier stage, tadpoles will be smaller and be at a competitive disadvantage in adulthood (Berven 1990, Kiesecker 1996).

This study investigated the effects of chronic low level MeHg ingestion on *X. tropicalis* tadpole metamorphosis. Other endpoints were monitored, such as abnormalities and mortality. Tadpoles were chronically exposed to MeHg at several developmental stages to determine if there was greater sensitivity at specific developmental periods. It was hypothesized that low concentrations of MeHg would alter the rate of metamorphosis.

3.2 Methods

3.2.1 Chronic exposure

Xenopus tropicalis is a model frog species of increasing importance, and was chosen for this study due to its rapid development. Adult *Xenopus tropicalis* were bred as previously described, and tadpoles were raised according to our protocol (Chapter 2). Tadpoles were staged before experimentation began (Nieuwkoop and Faber 1994) and kept separate in bowls to distinguish developmental stage differences to chronic exposure. The developmental stages used, tadpole apportionment and bowl arrangement can be seen in Table 3.1.

Experimental food was prepared as previously described for *X. tropicalis* (Chapter 2). Food was kept in aluminum foil wrapped 500mL dark glass bottles and 200mL of food was prepared weekly. Five grams of Sera Micron® were used in the food slurry to a density of 0.025g/mL. *X. tropicalis* food MeHg concentrations were 0, 50 and 500ng/g. Food was delivered using a 5mL latex-free syringe, and each bowl received 2mL of food daily. Water changes were carried out 6 days a week. Daily water change also involved bowls being scrubbed with a plastic scrub brush to remove any film or debris. Every 1.5L glass bowl was filled with approximately 1L clean, dechloraminated Aquatic Animal Care system water and covered with a sheet of Mylar-D™ to prevent UV-B wavelengths from the lighting from interfering with the experiment. Small air vents were cut in the covering to ensure adequate air circulation.

Tadpoles were maintained in a controlled environmental chamber (12:12 photoperiod; 24 ±2°C). Water was circumneutral (pH 7-7.4) with a conductivity of 158.6μS

Table 3.1 – Chronic study bowl setup with *Xenopus tropicalis* tadpoles. Number of tadpoles allocated and bowls used per initial developmental stage class are similar for all treatments.

Stage	Treatment					
	Control		50ng/g		500ng/g	
	Tadpoles	Bowls	Tadpoles	Bowls	Tadpoles	Bowls
46-50	30	6	33	6	34	6
51	18	3	18	3	18	3
52	11	2	12	2	12	2
53	9	2	10	2	10	2
54	18	3	18	3	18	3
54^{+}	12	2	12	2	12	2
Total	98	18	103	18	104	18

and a hardness of 30-40mg/L $CaCO_3$. MeHg content of the water was below the detection limit (<0.02ng/L).

Observations were taken daily. Dead animals were removed, placed in clean 15mL Falcon tubes and kept for methylmercury analysis. Any physical abnormalities and behavioural changes were noted. Lethargy was tested by visual inspection for swimming behaviour. If no swimming was observed and a lack of general movement was evident, a startle response test was applied through gentle physical stimulation of the tail with a disposable plastic pipette. Lethargy measurement was based on the need for stimulation to begin swimming behaviour. Abnormalities were noted descriptively.

Once forelimb emergence occurred, juveniles were anaesthetized using 1g/L MS-222 and dissected using microscissors and fine tipped forceps under a Leica Zoom 2000 dissection microscope at 20 to 45X magnification. The hypothalamus and tail were removed and quick frozen on dry ice. The remainder of the body was freeze dried for MeHg analysis. Tails were freeze dried for total Hg (totHg) analysis.

3.2.2 Mercury Analysis

MeHg body burden analysis was carried out as previously described (Chapter 2). Tails from dissections and 5% of tadpoles from the final day cull were chosen to provide a subset of 7% of all tadpoles for totHg analysis. TotHg analysis was carried out using a Nippon Instruments Corporation SP-3D Total Hg Analyzer. Samples were combusted at 800°C and mercury was catalytically reduced to Hg^0 followed by dual gold amalgamation and detection using cold vapour atomic absorption. A small mass (~0.005g) was cut from the freeze dried tadpole tail, placed in a clean, ceramic boat and prepared with the reagent

powders M (1:1 $Ca(OH)_2$:Na_2CO_3) and B (Al_2O_3) in the layered pattern: powder 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 tube and analyzed on setting 2 for tissue analysis, with the measurement range adjusted according to the treatment level the tadpole received. A standard reference material (DORM-2) was run for every 7 samples. Blanks were also run with check standards, and replicates were run for 95% of all samples.

Statistical analyses was performed using Microsoft Excel and hand calculations.

3.3 Results

Experimentation was terminated after 78 days, when 90% of the 500ng/g tadpoles had been removed from the study through death or metamorphosis. The remaining 10% of tadpoles were all showing abnormalities associated with MeHg ingestion found in the toxicity experiment (Chapter 2) and were extremely lethargic.

3.3.1 Mortality

All treatment concentrations were below both our 33 day and 42 day LC50s for *X. tropicalis*, 926ng/g and 643ng/g respectively, calculated from our toxicity studies (Chapter 2), however, more than 50% mortality occurred in the 500ng/g treatment (Figure 3.1A). There was little difference between the two MeHg treatments and the Control treatment until after day 30 when mortality began to increase. Mortality in the 500ng/g treatment increased steadily after day 38-39, with 143% higher mortality than that in the Controls by day 77. Mortality in the 50ng/g treatment was 69% of that in Controls by the end of the experiment.

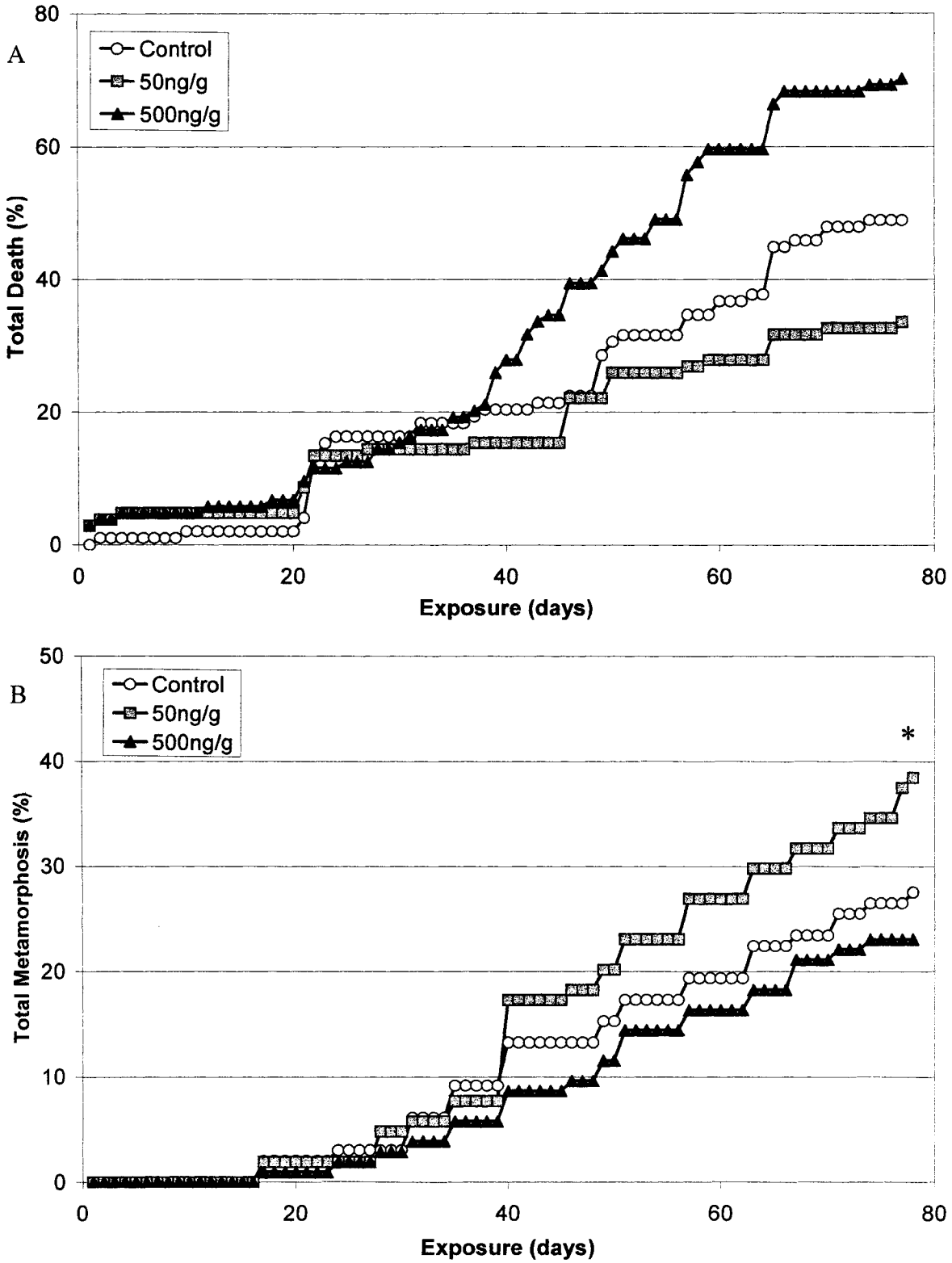

Figure 3.1 – A) Mortality over time in the *X. tropicalis* chronic MeHg exposure. B) Metamorphosis over time in the chronic MeHg exposure. Metamorphosis in the 50ng/g treatment was significantly different from Controls ($p<0.03$).

3.3.2 Metamorphosis

Metamorphosis was 140% higher in the 50ng/g treatment than the Controls and 167% higher than the 500ng/g treatment (Figure 3.1B). Testing by Pearson's Chi square revealed that this is a significant difference ($p<0.03$). Metamorphosis in the 500ng/g treatment was 84% of that in the Controls. By day 40, metamorphosis in the 50ng/g treatment had exceeded that in Controls and the 500ng/g treatment and continued to increase until experimentation ended. The 500ng/g treatment had low metamorphosis throughout the experiment, never exceeding either the 50ng/g treatment or the Controls.

3.3.3 Deformities and Abnormalities

The most common abnormality was a combination of lethargy and inversion in the highest treatment level. A second behavioural abnormality of tadpoles remaining at the water surface was designated surface resting. These two abnormalities were noted consistently in the 500ng/g treatment, with fewer observations in the other two treatments. Emaciation was rarely observed and without a clear trend. This abnormality was usually paired with an overall colouration change to a lighter or darker shade than normal tadpole colouration. Day of onset and proportions of abnormalities are presented in Table 3.2. Other abnormalities noted in only a few individuals included three tadpoles in the 500ng/g treatment with difficulties swimming, three tadpoles in the 500ng/g treatment visibly struggling to maintain an upright posture, one tadpole in the 500ng/g treatment with one extremely reduced hind limb and one normally developed hind limb, and one tadpole in the 500ng/g treatment maintaining a vertical posture in the water column with its mouth at the water surface. A total of three tadpoles combined in the Control and 50ng/g treatments were

Table 3.2 – Proportions of deformities and abnormalities seen in *X. tropicalis* chronic MeHg exposure. Day of initial observation is indicated. Once an abnormality was noted, it continued to be observed until experimentation ended.

Test level	**Lethargy/Inversion**	**Surface Resting**	**Colouration**	**Emaciation**
Day	12	12	12	16
Control	0.23	0.20	0.07	0.08
50ng/g	0.25	0.23	0.01	0.03
500ng/g	0.64	0.52	0.06	0.06

observed to have abdominal bloating and regional distensions of the gut, and no instances of these abnormalities were noted in the 500ng/g treatment. Only four Control tadpoles were observed to have kinking of the tail. As these abnormalities were deemed low level and not universal, they were not included in Table 3.2.

3.3.4 Body Burden

Quality control and quality assurance samples were run during the MeHg digestions. Blanks were below the detection limit (<0.02pg/g) and contributed no MeHg to samples. Average standard reference material recovery was 120±6% of the expected value, which is acceptable but elevated. Ideally, recovery values would fall between 90 and 110%. Since the blanks contributed no MeHg to samples, it is unlikely this increased recovery is due to contamination. Average percent difference between replicates was 9.7±7.5%. Quality control and quality assurance samples were run during the totHg analyses. Blanks contributed an average of 0.04±0.02ng Hg to samples. Average standard reference material recovery was 90±3.9% of the expected value. Average percent difference between replicates was 7.5±4%.

MeHg body burden analyses were performed on 93% of tadpoles. Body burdens increased as treatment concentrations increased (Figure 3.2A). When compared to totHg concentrations, it is noted that body burdens fall within the same orders of magnitude for both MeHg and totHg concentrations (Figure 3.2B). Percentage of MeHg within totHg body burdens ranged from 10 to 141% across all treatment levels. For matched samples, totHg body burdens were 85±4%, 87±20% and 20±3% MeHg in Controls, 50ng/g and 500ng/g, treatments respectively.

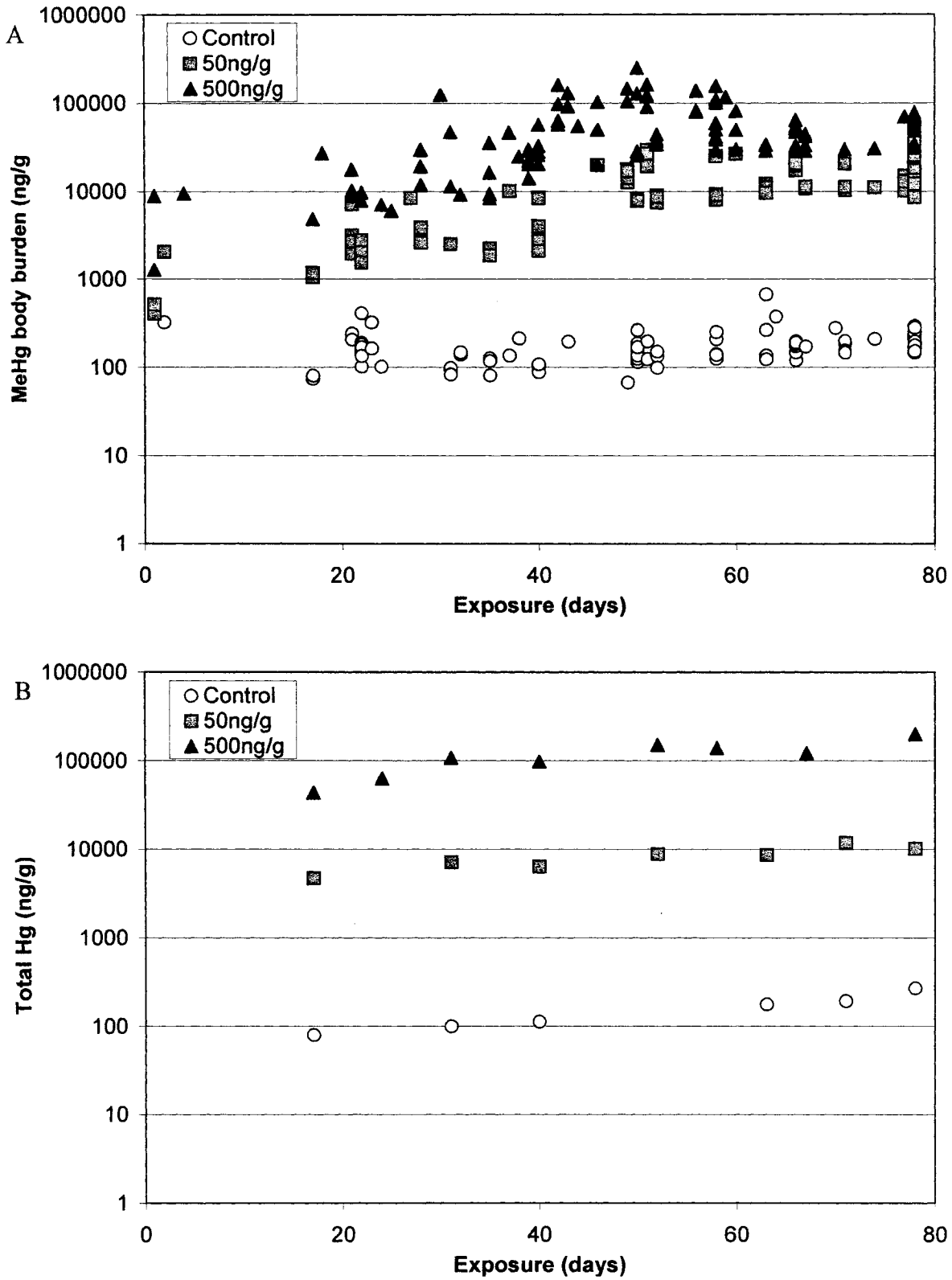

Figure 3.2 – Body burden measurements for *X. tropicalis* chronic MeHg exposure study. N=300. A) MeHg body burdens. B) Total Hg body burdens from a subset of tadpoles from the study.

Control tadpoles contained relatively low levels of MeHg, with little increase over the course of the study (Figure 3.3). Analysis of tadpoles from the 50ng/g treatment showed an increase in MeHg body burden over time with day 78 body burdens ranging from approximately 10 000ng/g to 55 000ng/g dry wt. (Figure 3.4). MeHg body burden in the tadpoles in the 500ng/g treatment increased over time up to approximately day 50 and seemed to decrease in MeHg over the final 20 to 28 days of experimentation, with body burdens below 100 000ng/g dry wt. for the final 18 days (Figure 3.5). A time lag also seems to occur in the increase in body burden for both 50ng/g and 500ng/g treatments, as the body burden remained relatively low initially and increased after day 40.

3.3.5 Developmental stage differences

Differences in average mortality and metamorphosis were observed depending on initial developmental stage when experimentation began. Stages used were NF stage 46-50, 51, 52, 53, 54 and 54^{+}, which encompassed all tadpoles between stage 54 and metamorphic climax (Table 3.1). In all treatment levels, high metamorphosis and low mortality occurred in NF stage 54^{+} (Figure 3.6A). The highest mortality in all treatments was observed at NF stage 52 (Figure 3.6B). 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. Percent metamorphosis was higher in the 50ng/g treatment for NF stages 51 to 54^{+} than the Controls. All tadpoles removed from the 50ng/g NF stage 53 and 54^{+} bowls were metamorphs. Within the Controls, the highest percent metamorphosis was observed in NF stages 53 and 54^{+}, while the highest mortality was observed in NF stages 52

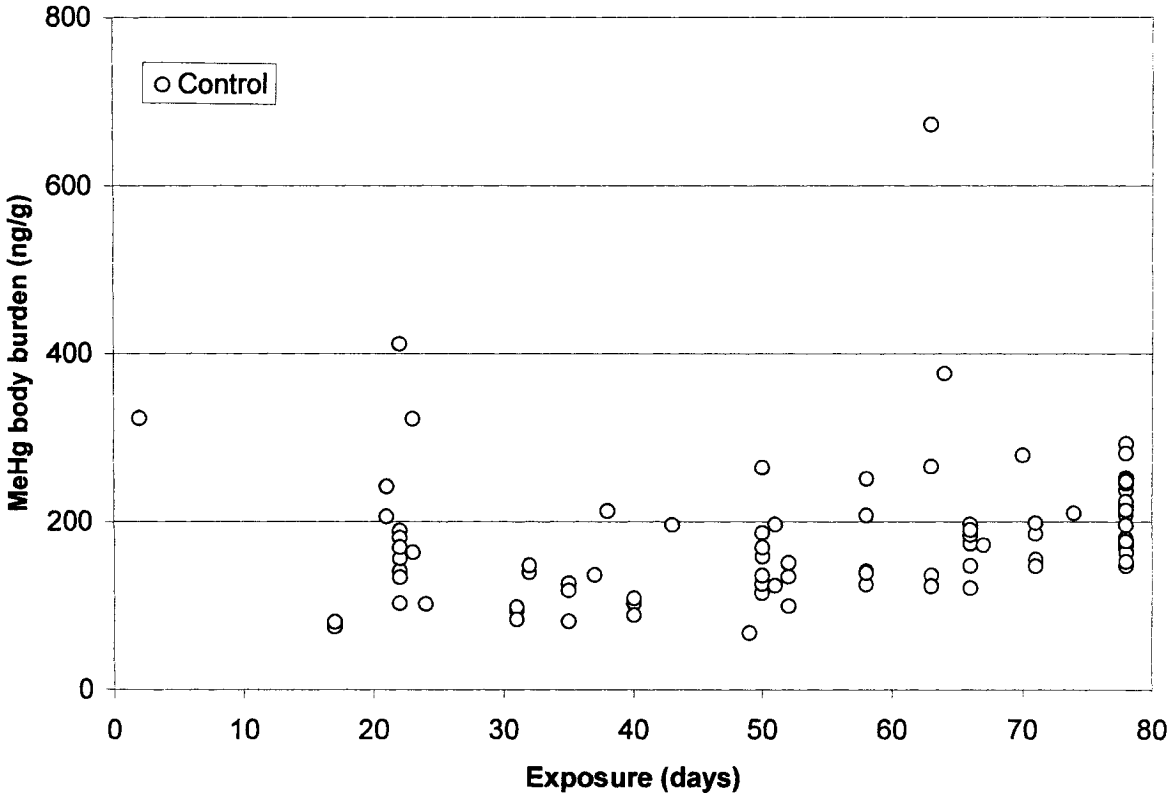

Figure 3.3 – Body burden of MeHg in Control treatment tadpoles over the course of the *X. tropicalis* chronic MeHg exposure experiment. N=98.

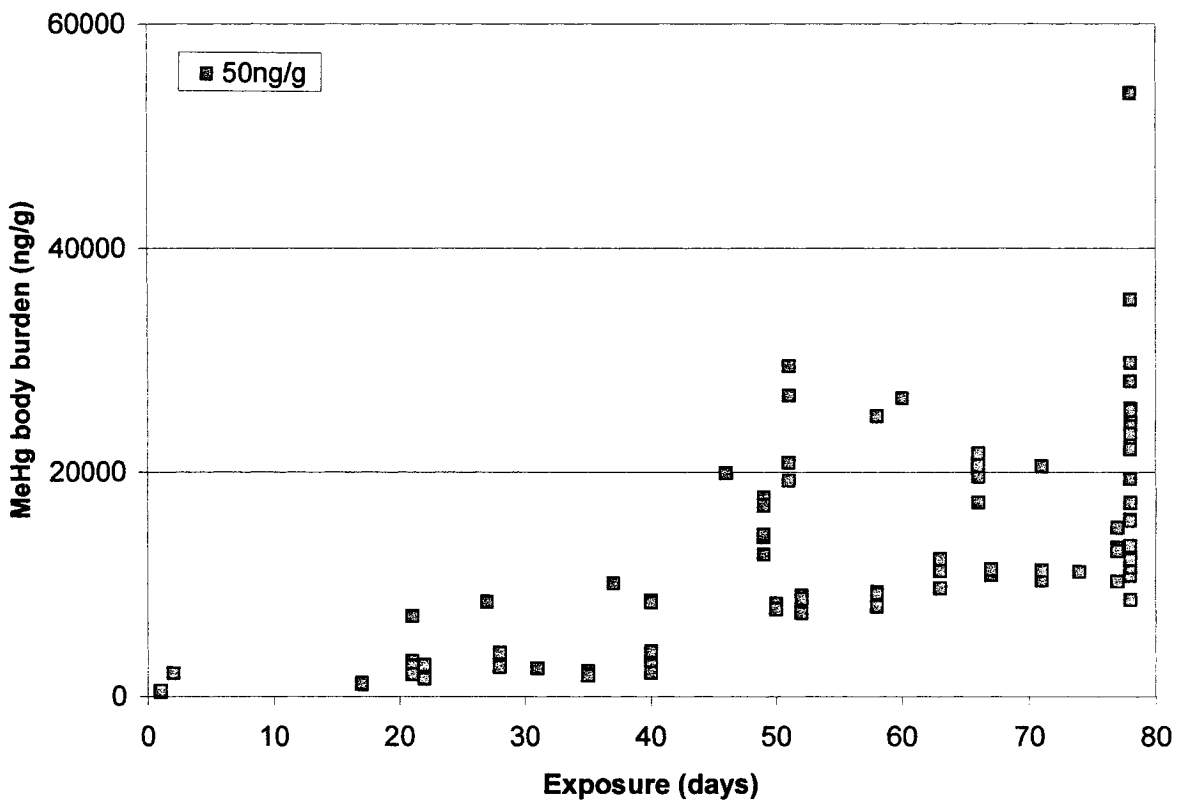

Figure 3.4 – Body burdens of MeHg in 50ng/g level tadpoles over the course of the *X. tropicalis* chronic MeHg exposure experiment. N=103.

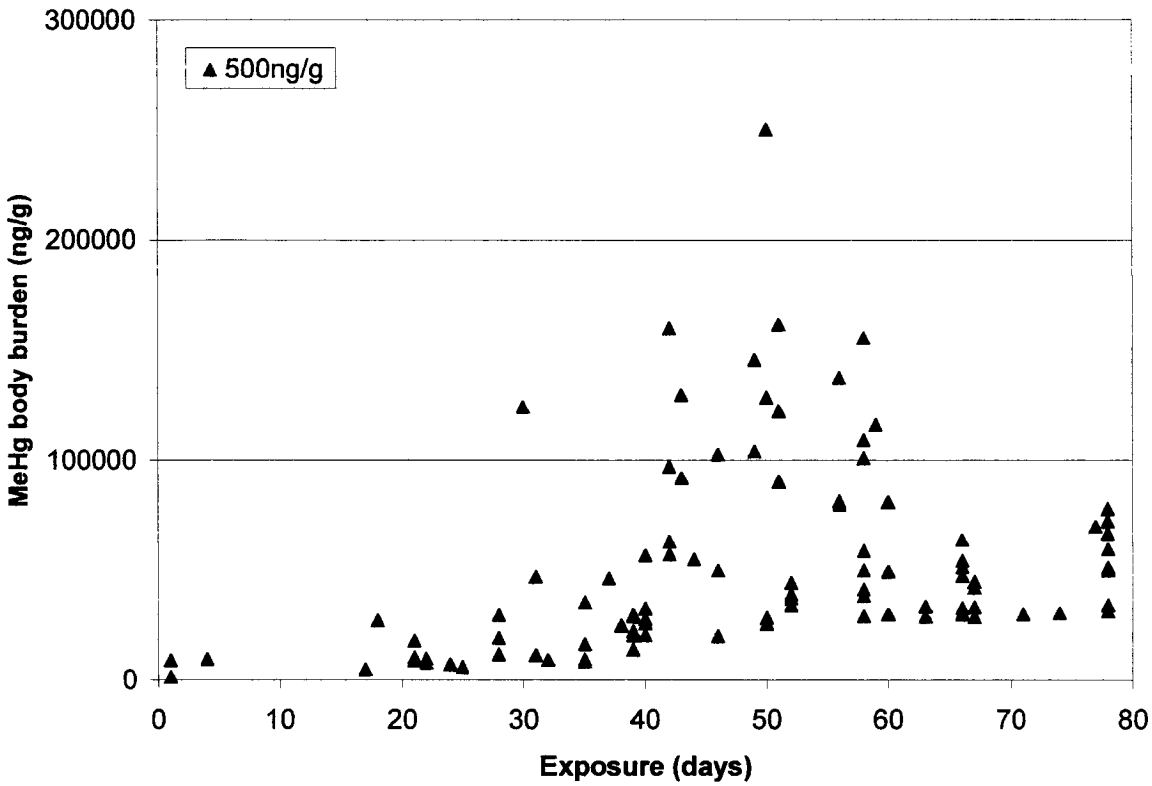

Figure 3.5 – Body burdens of MeHg in 500ng/g level tadpoles over the course of the *X. tropicalis* chronic MeHg exposure experiment. N=104.

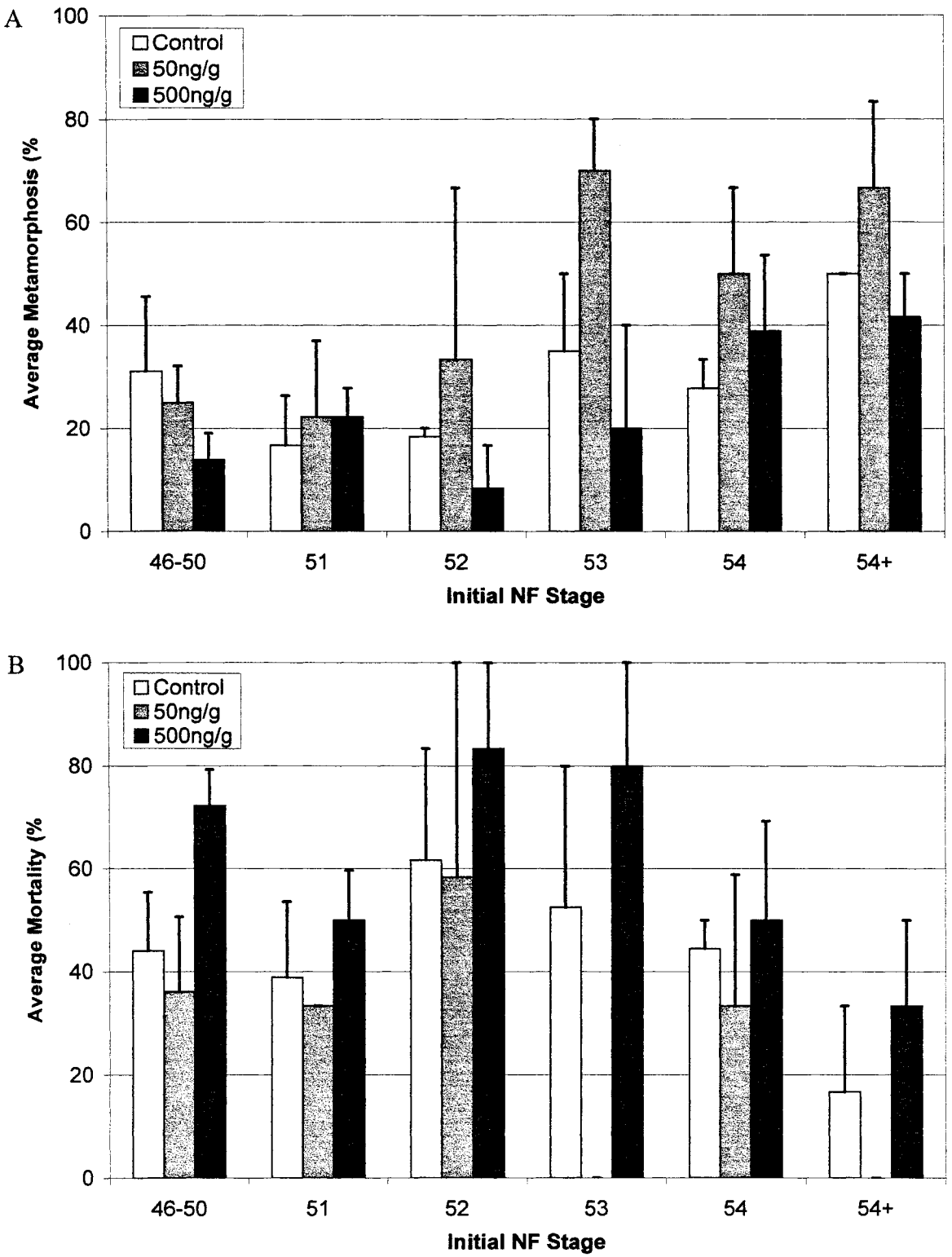

Figure 3.6 – Final differences in response with initial Nieuwkoop-Faber developmental stage class in the *X. tropicalis* chronic MeHg exposure study. A) Average metamorphosis with standard error. B) Average mortality with standard error. For N values, see Table 3.1.

and 53. More metamorphosis occurred in the most advanced NF stages 54 and 54^{+} in the 500ng/g treatment.

3.3.6 Body weights

Average body weights were calculated for all metamorphosed tadpoles in all treatment levels and Controls (Table 3.3). All body weights are within one standard error measurement and not considered statistically different.

3.4 Discussion

More than 50% mortality occurred over the course of the experimental exposure to MeHg, despite choosing MeHg concentrations below the two LC50s calculated in the previous chapter for this species. This is not necessarily unexpected, however, since it was seen that over the course of the previous exposure, the LC50 estimate declined over time (Chapter 2). The duration of this experiment was longer than previous toxicity tests, as it was designed to investigate chronic exposure to MeHg, therefore tadpoles had a longer period during which to experience MeHg toxicity. Death also occurred within the low concentration and the Control (Figure 3.1A). The mortalities in the Controls are a result of natural attrition, as there was no obvious cause of death. The difference between Control and 50ng/g deaths is almost equal to the increase in metamorphosis in the 50ng/g treatment over the Controls (Figure 3.1B).

The 50ng/g treatment had 38.5% metamorphosis compared to 27.5% in the Controls, which was found to be significantly different. This observation supports the initial

Table 3.3 – Average wet weight and dry weight for metamorphosed tadpoles in the *X. tropicalis* chronic MeHg exposure. Standard error is given, as well as the number of metamorphosed tadpoles from each test level. All analyses were based on dry weight measurements. Wet weights were taken before dissection. Tadpoles were patted dry with a Kimwipe to remove excess water. Dry weights were taken after freeze drying, before MeHg body burden analysis.

Test level	**N**	**Wet wt.**	**SE**	**Dry wt.**	**SE**
Control	27	0.70	0.06	0.075	0.005
50ng/g	40	0.63	0.03	0.066	0.002
500ng/g	24	0.60	0.04	0.068	0.004

prediction that low level MeHg may act as a stressor which could increase the rate of metamorphosis. A higher metamorphic rate results in a lower mortality rate for the 50ng/g treatment, as tadpoles are able to escape the toxic environment before the damage caused by MeHg is lethal. This result is also supported by Unrine et al. (2004), who found an increase in metamorphosis, measured by a decrease in days to hindlimb and forelimb emergence in *R. sphenocephala* tadpoles ingesting a diet of 3298 ng/g dry wt. inorganic Hg, with a measured level of 49.6ng/g dry wt. MeHg. Our study shows that tadpole metamorphosis increased significantly with exposure to 50ng/g MeHg alone, which is an important result because tadpoles in their natural environment are likely exposed to this level of MeHg. For example, in this study, we measured environmental MeHg body burdens in tadpoles ranging from 1 to 126ng/g and 14 to 140ng/g in insects (Chapter 2). Additionally, Plourde et al. (1997) measured between 10 and 20ng/g dry wt. MeHg in suspended particulate matter and 60 to 170ng/g dry wt. MeHg in zooplankton in natural lakes in Quebec. Westcott and Kalff (1996) measured an average of 95ng/g dry wt. MeHg in zooplankton in clear water lakes and an average of 289ng/g dry wt. MeHg in zooplankton from brown water lakes in south central Ontario. Unrine and Jagoe (2004) found MeHg levels ranging from 8 to 57ng/g dry wt. MeHg in periphyton collected from several environmental sites, including a constructed wetland, in South Carolina, USA. Due to the detrivorous nature of tadpoles, all of these ecosystem components could become a part of a tadpole's diet, and these concentrations are found in the environment in North America.

The highest rate of metamorphosis occurred in the 50ng/g treatment when exposure began at NF stages 51 to 54^{+} (Figure 3.6a). The greatest amount of metamorphosis occurred in the Controls at NF stages 46-50. This difference in the rate of metamorphosis may

indicate a developmental sensitivity. Tadpoles exposed to MeHg at an earlier developmental stage may not experience the same stress response as those exposed later in metamorphosis. Bridges (2000) showed that when *R. sphenocephala* tadpoles were exposed to the pesticide carbaryl throughout development, they metamorphed later than those treated at specific life stages. In our study, metamorphosis was lower in four out of six developmental stage groups in the 500ng/g treatment compared to Controls, and mortality was elevated in all developmental stage groups in the 500ng/g compared to both Controls and 50ng/g treatments. The rate of metamorphosis seemed similar to that of Controls but slightly lower in the 500ng/g treatment, and seemed relatively independent of the increasing mortality rate (Figure 3.1). This supports the second part of the initial hypothesis, that high concentrations of MeHg would suppress metamorphosis. The lower concentrations of MeHg in the 50ng/g treatment seem to be acting as an environmental signal, increasing the rate of metamorphosis, whereas the higher concentration in the 500ng/g causes the tadpoles to accumulate too much MeHg, and tadpoles die before metamorphosis is complete. Although the mechanism of this change in metamorphic rate was not measured, we suggest that the tadpole is stressed by the toxic actions of MeHg and a hormonal response is occurring which is changing the rate of development. This should be a focus of future research.

According to Figure 3.6, the tadpoles most likely to undergo metamorphosis are those exposed to MeHg later in development. This is probably partially due to the reduced amount of time required for metamorphosis completion, as development had already started. Tadpoles exposed to MeHg at an earlier developmental stage are exposed to MeHg longer than those exposed at a later developmental stage, increasing the probability of mortality due to direct toxicity or through the symptoms of MeHg poisoning. In a natural pond, stressors

such as pond drying would be most effective in increasing metamorphic rate at later metamorphic stages. At early developmental stages, intense stressors may not increase the rate of metamorphosis because the tadpole body is not competent to respond. It has been shown that an increase in the stress hormone corticosterone in early development can cause a decrease in the developmental rate (Hayes et al. 1993).

It is also possible that the energy the tadpole had to expend repairing the damage caused by MeHg and attempting to depurate MeHg has a suppressive effect on energy partitioning towards metamorphosis early in development. Later in development, the body could have enough energy reserves to experience an increase in metamorphic rate due to the toxic stress of MeHg as well as attempt to excrete it. This energy usage would be an increased maintenance cost to the tadpole, as has been discussed by Rowe et al. (1998). *R. catesbeiana* tadpoles from sites polluted with trace elements from coal ash had elevated metabolic rates compared to those from an unpolluted reference site. They hypothesized that this was due to the body requiring more energy for basic physiological processes due to the environmental stress. The result is less energy for other less crucial processes to immediate survival such as growth and development. This energetic hypothesis fits the developmental results of this study however the dry wt. body weights are not different enough to suggest an effect on growth (Table 3.3). But the amount of energy expended on maintaining the tadpole body after MeHg exposure and the attempts to transform and excrete MeHg may have reduced the overall amount of energy available to tadpoles for metamorphosis.

The biological significance of an increase in metamorphic rate lies in the timing of this increase. If the increase occurs early in development, before the tadpoles grow to a size which allows them to compete in the juvenile and adult world, then juvenile survival will

decline. This has been seen in other studies with chemicals toxic to tadpoles (Berven 1990, Kiesecker 1996, Marco and Blaustein 1999). If the increase occurs later in development, the tadpoles could be experiencing other developmental issues from the insult of MeHg on *de novo* tissue formation, such as gonadal development problems with MeHg possibly interfering with steroid action (Friedmann et al. 2002, Hammerschmidt et al. 2002, Martin et al. 2003). Another possible action of MeHg on developing tissues would be the inhibition of microtubules and mitochondrial function. Additionally, the effects of MeHg on motor control and lethargy or feeding drive may also handicap new juveniles. In our study, the increase in metamorphosis was observed in later development and tadpoles exposed to MeHg from early stages throughout development had low rates of metamorphosis. Therefore these tadpoles may have successfully completed the transition to adulthood, but there may be effects on their success as adults due to the MeHg stress during development.

There was an increase in body burden concentration with respect to the concentration being ingested (Figure 3.2). There was also an increase in the concentrations measured in tadpoles after day 40 in the 50ng/g (Figure 3.4) and 500ng/g treatment, although the 500ng/g treatment tadpoles appeared to decrease in measured body burden after day 50 (Figure 3.5). The additional measurements of totHg showed that the 500ng/g treatment had a lower proportion of MeHg (0.2) than the 50ng/g treatment or Controls (~0.86). Perhaps more energy is being utilized by the tadpole to transform MeHg to inorganic Hg for excretion, which would be an essential physiological process in this highly contaminated treatment (Rowe et al. 1998). This could lead to a reduction in development and the differences in metamorphosis between the 500ng/g treatment and the 50ng/g treatment, where higher proportions of MeHg are found within the body yet more metamorphosis is occurring. The

50ng/g concentration may be low enough to act as a stressor, but the tadpole can allocate more energy towards metamorphosis and less toward the excretion of MeHg. This energy allocation could also explain the decrease in MeHg body burden towards the end of the experimentation in the 500ng/g treatment.

Deformities seen in the previous experiment such as kinking and abdominal deformities occurred in only a few individuals in this study. The most common abnormalities involved lethargy, usually combined with inversion and surface resting. This combination had not been seen previously, but was present in 64% and 52% of 500ng/g tadpoles, respectively. Additionally, although emaciation was noted, the numbers were not suggestive of a MeHg mediated loss of the drive to feed, and did not follow a MeHg related trend. Most abnormalities involved the behavioural modifications such as lethargy and inversion, which are still attributable to the effects of MeHg within the brain (Aulerich et al. 1974, Wren et al. 1987).

This study shows a significant change in *X. tropicalis* metamorphic rate due to the ingestion of low level, environmentally relevant concentrations of MeHg (Figure 3.1B). A potential change in the bioaccumulation of MeHg within tadpoles was dependent on the concentration ingested as well (Figure 3.5). The results support the initial hypothesis that lower concentrations of MeHg would act as an environmental stressor, thereby increasing the rate of metamorphosis, whereas the higher concentrations would suppress metamorphosis and increase mortality. We show that amphibians are currently exposed to levels of MeHg that can alter behaviour and development. Inputs to the global Hg cycle through fossil fuel combustion and industry further pollute areas where amphibians grow and develop. If MeHg has the ability to disrupt hormonal processes controlling amphibian

metamorphosis, there is reason for concern for human populations as well. Amphibians are models of fetal development, since many systems are well conserved across taxa. We show that MeHg can change the rate of metamorphosis and affect the behaviour of tadpoles when ingested at environmentally relevant concentrations, therefore fetal development could also be affected not only by the neurotoxicity of MeHg, but the ability of MeHg to modulate the hormones controlling development. Body systems of future research interest in this field include hormonal systems, metabolism and detoxification mechanisms.

Chapter 4

4.0 Summary and Conclusion

4.1 General Conclusions

In this study, we showed that dietary MeHg is toxic to amphibian tadpoles and can cause changes to behaviour and development at environmentally relevant levels. This is the first study of its kind, as amphibian MeHg toxicity studies in the past have been performed using elevated MeHg concentrations in water. This is not an environmentally relevant delivery method, since MeHg in natural waters is usually in the ng/L range and MeHg in the food chain is 10^3 times higher. Only one other study has investigated dietary Hg effects on amphibians, but both inorganic Hg and MeHg were used (Unrine et al. 2004).

4.1.1 Toxicity studies

In summary, we observed a species difference between the North American amphibian species, *B. americanus* and *R. pipiens*, and the African frog model *X. tropicalis*. The *B. americanus* and *R. pipiens* G25 tadpoles were significantly more sensitive to dietary MeHg than the equivalent stage *X. tropicalis*. This was determined through the examination of LC50 estimates calculated after 33 days of dietary MeHg exposure. It was also determined that more developmentally advanced *B. americanus* G27-30 tadpoles were significantly less sensitive to dietary MeHg as compared to the *B. americanus* G25 tadpoles. The abnormalities most often observed in these toxicity studies were lethargy, inversion and emaciation. Lethargy occurred in 100% of 1000ng/g treatment tadpoles for all species. These results can be directly related to piscivorous wildlife studies showing MeHg poisoning symptoms in mink which include lethargy, loss of motor control, and loss of eating drive

(Aulerich et al. 1974, Wren et al. 1987). Body burdens increased over time in all tadpoles. These results indicate that dietary MeHg will accumulate in tadpoles to a high degree, relative to the concentrations being ingested.

4.1.2 Chronic study

The most notable result from the chronic exposure of *X. tropicalis* was the finding that metamorphosis was significantly increased ($p<0.03$) by 140% in the 50ng/g treatment compared to Controls, resulting in a reduction of mortality observed in the 50ng/g treatment compared to Controls as well. Metamorphosis was also suppressed in the 500ng/g treatment, as mortality increased by 143% over Controls. This shows that MeHg has the ability to affect the development of tadpoles at environmentally relevant concentrations. Tadpoles were less likely to metamorphose from the early stage exposures compared to late stage exposures in all treatments. Similar abnormalities were observed in the chronic exposure as were seen in the toxicity studies. The lethargy/inversion combination was the most common abnormality, observed in 64% of 500ng/g treatment tadpoles. Surface resting, a new symptom, was also observed in 52% of 500ng/g treatment tadpoles. These motor control and behavioural changes are similar to MeHg poisoning symptoms observed in mink (Aulerich et al. 1974, Wren et al. 1987). The body burden data also revealed a possible difference in MeHg accumulation in tadpoles as concentration increases, as the tadpoles in the 500ng/g treatment appear to reach an upper limit of accumulation. After 50 days of increasing body burden, all the remaining tadpoles to metamorphose or die in the last days of experimentation had body burdens below 100 000ng/g dry wt., whereas at day 40, the tadpoles removed from the 500ng/g treatment exceed 100 000ng/g dry wt. A similar change

in body burden was observed in the 50ng/g treatment at day 40, but tadpoles continued to increase in body burden until the experiment ended.

4.1.3 Environmental sampling

Upon examination of environmental samples from Eastern Ontario and Western Quebec as well as values in the literature, the test levels used fall within values measured in nature (Figure 4.1). From the 1ng/g to the 100ng/g test level, MeHg concentrations in nature occur in ecosystem components tadpoles would access as food. Detrivores grazing on periphyton and detritus exceed 3ng/g which we calculated to be the conservative tolerable intake level from the 33 day LC50s for *B. americanus* and *R pipiens* G25 toxicity studies. Seston, phytoplankton and suspended particulate matter exceed the 10ng/g test level. Conspecifics, which have been observed being eaten (Gibson, pers. obs.), exceed the 10ng/g test level and range past the 50ng/g and 100ng/g test levels. Insects and zooplankton also range well past the 100ng/g treatment. These food concentrations have been shown to cause obvious behavioural abnormalities as well as changes in development. For example, at 10ng/g and 100ng/g, 21 and 11% of tadpoles were emaciated in the *B. americanus* G27-30 toxicity study, respectively. In the chronic study, the 50ng/g treatment increased metamorphosis by 140% over Controls. This concentration falls between the environmental concentrations measured for seston and zooplankton.

4.1.4 Conclusion

This study uses food concentrations of MeHg which are comparable to MeHg concentrations found in the natural environment, and now are shown to detrimentally affect

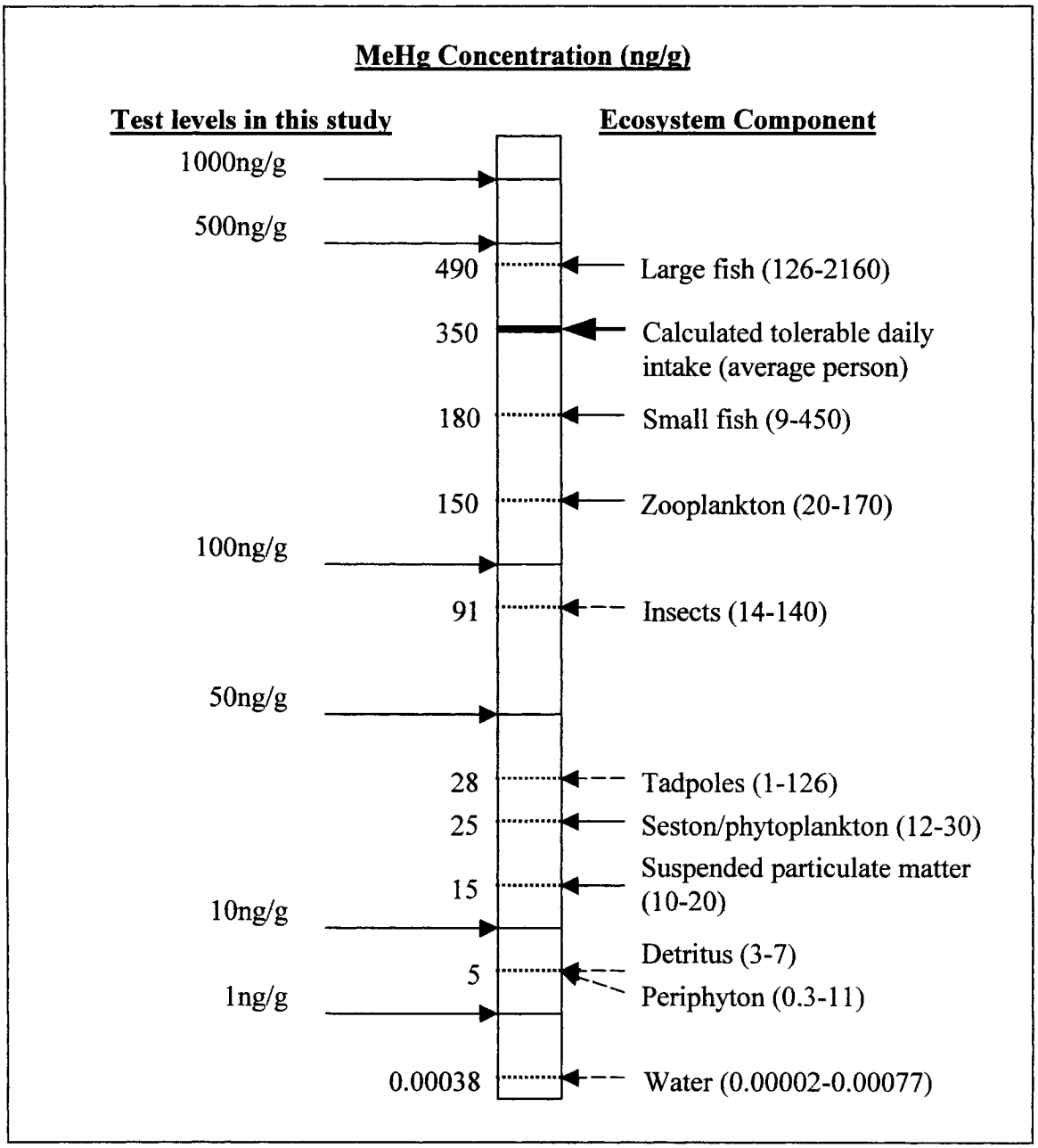

Figure 4.1 – Revised general schematic of environmental MeHg concentrations. All concentrations are given on a dry weight basis, where applicable. Data gathered from the present study is indicated by the dashed arrows. Test levels for this study are shown in the diagram with arrows indicating where they fall within environmental concentrations. Mean environmental concentrations are given on the left hand side of the ruler with ranges provided in parentheses on the right hand side of the ruler. A calculated daily consumption guideline is indicated by the heavy black ruling, adapted from the provisional tolerable weekly intake published by JECFA (2003). Literature values adapted from Hall et al. (1997), Hickey et al. (2005), Plourde et al. (1997), Unrine and Jagoe (2004), Watras and Bloom (1992), Westcott and Kalff (1996), Wren et al. (1986).

tadpole behaviour and development. Induction of metamorphosis as a survival mechanism occurs at the natural food levels between seston and zooplankton. The rate of metamorphosis increases because MeHg is an environmental stress. MeHg will eventually bioaccumulate to levels that will kill the tadpole, either directly or by inhibiting its ability to survive predation through the symptoms of MeHg poisoning. Small changes in a tadpole's ability to avoid predation can severely affect survival to adulthood. The question remains, however, whether the increased rate of metamorphosis under MeHg stress allows the tadpole to develop well enough to survive in adulthood.

As Hg is released from anthropogenic sources the amount of Hg available in the environment for methylation increases. This can increase the amount of MeHg in the food chain, thus affecting all organisms. At high concentrations of MeHg, some tadpoles showed resistance to the toxic effects, but 500ng/g caused 70% mortality for the chronic exposure *X. tropicalis*. At day 40, we observed a change in strategy between the two chronic MeHg exposure treatments. The rate of metamorphosis increased and the mortality rate decreased in the 50ng/g treatment, whereas the mortality rate increased and the rate of metamorphosis was suppressed in the 500ng/g treatment. Tadpoles in both treatments were carrying higher body burdens after day 40. Further research should investigate the sublethal effects of MeHg on tadpoles, as MeHg effects on amphibian development will lead to questions about the sublethal effects of MeHg on human development.

4.2 Future Research Considerations

As this is the first study to focus on dietary MeHg effects on amphibian metamorphosis, this field is completely open to investigation. The results of this study lead

to important questions about the mechanisms behind the effects observed.

A low MeHg concentration, chronic exposure study with stage specific dissections to measure metamorphic hormone levels should show what hormones are being affected (Figure 1.1). The hormones of immediate interest would be CRF, the thyroid hormones, T_4 and T_3, as well as corticosterone. Gene expression assays could also be performed to examine what genes controlling the metamorphic cascade are being affected by MeHg ingestion, specifically whether the deiodinases are being affected in larval amphibians in the same way that has been shown in fetal mice (Watanabe et al. 1999). As microarrays are available for *X. tropicalis* (Chalmers et al. 2005), other target genes could also be identified which may be affected by MeHg, such as specific genes involved with organogenesis and apoptosis (Monetti et al. 2002).

Since we have shown that MeHg ingestion causes similar behavioural abnormalities in tadpoles as have been observed in MeHg poisoning cases in mink (Aulerich et al. 1974, Wren et al. 1987), the specific tissue damage incurred by MeHg on tadpoles should be investigated. Due to the paucity of studies done on amphibians with respect to MeHg, many observations have to be explained by extrapolating from effects observed in different taxa. Classical histological examinations of the brain, liver, and interrenal glands of experimentally exposed tadpoles would provide some insight into the direct effects of MeHg on amphibian larval tissue. It could also be informative to examine whether tissue damage occurs differently as metamorphosis progresses, as tadpole metamorphosis involves widespread tissue remodeling and MeHg may affect the ability of microtubules to complete cellular processes (Hunter and Brown 2000).

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APPENDIX

Table 1 – Validation data for study design. Waste water was filtered to remove detritus and filters, waste water and feces removed during a regular cleaning were analysed for MeHg content. ND – non-detectable.

Samples	**Level (ng/g)**	**Average MeHg**	**Comment**
Filters		**(ng/g)**	
FWW003	Control	6.07	Aug 6/03
FWW004	1ng/g	23.08	Aug 6/03
FWW005	10ng/g	15.03	Aug 6/03
FWW006	100ng/g	151.68	Aug 6/03
FWW007	1000ng/g	1497.35	Aug 6/03
FWW009	Control	7.94	Aug 25/03, after weekend
FWW010	Control	1.79	Aug 27/03, Wednesday
FWW011	1ng/g	ND	Aug 25/03, after weekend
FWW012	1ng/g	11.62	Aug 27/03, Wednesday
FWW014	10ng/g	ND	Aug 25/03, after weekend
FWW015	10ng/g	17.14	Aug 27/03, Wednesday
FWW016	100ng/g	11.67	Aug 25/03, after weekend
FWW017	100ng/g	185.19	Aug 27/03, Wednesday
FWW018	1000ng/g	198.76	Aug 25/03, after weekend
FWW019	1000ng/g	1245.31	Aug 27/03, Wednesday
Feces		**(ng/g)**	
FC001	Control	26.11	Aug/04
FC002	1ng/g	192.30	Aug/04
FC003	10ng/g	286.61	Aug/04
FC004	100ng/g	3539.38	Aug/04
Waste Water		**(ng/L)**	
WW001	Control	0.31	Aug/03
WW002	1ng/g	0.52	Aug/03
WW003	10ng/g	2.33	Aug/03
WW004	100ng/g	21.24	Aug/03
WW005	1000ng/g	241.63	Aug/03
Food		**(ng/g)**	
F001	Tadpole granules	27.24	*R. pipiens, B. americanus* studies
F002	Sera Micron	14.14	*X. tropicalis* study