CHRONIC DIETARY METHYLMERCURY EXPOSURE ON THREE JUVENILE STAGES OF THE CRAYFISH *PROCAMBARUS CLARKII*

by

HEATHER A. BRANT

(Under the Direction of CHARLES H. JAGOE)

ABSTRACT

The red swamp crayfish (*Procambarus clarkii*) is an opportunistic omnivore, feeding on algae, detritus, and animal matter. The contribution of each of these to the diet varies with availability as well as age and sex of the crayfish. Few studies have addressed how sex and age affect Hg uptake and elimination in crayfish, or examined physiological and behavioral changes due to chronic dietary Hg exposure. We exposed juvenile crayfish (n=72) of known age and sex to diets containing relatively high and low concentrations of methylmercury (MMHg) using a 2x2x3 randomized block design. The low Hg diet averaged 9 ppb Hg fresh weight (80% MMHg) while the high Hg diet averaged 278 ppb Hg fresh weight (98% MMHg), both environmentally-realistic concentrations. Sub-lethal effects after 142 days of chronic exposure included alterations in behavior and growth, with severity varying based on sex and age. In nature, these effects could reduce crayfish survival.

INDEX WORDS: mercury, crayfish, behavior, chronic

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DEDICATION

In dedication to WLS, for your patience and motivation

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TABLE OF CONTENTS

Page

CHAPTER 1

INTRODUCTION AND OVERVIEW

INTRODUCTION

Over the last century, pollution has become an increasingly important anthropogenic environmental factor that can influence species distribution, abundance, and survival. One such pollutant is mercury (Hg). Environmental laws and regulations have been adopted to protect biota from such stressors. However, most regulations, especially those concerning allowable or tolerable pollutant concentrations in the environment, are built largely upon data from acute laboratory tests. In many instances, such data lacks true environmental relevance.

Concentrations of Hg in any particular aquatic system have potential to reach acute concentrations that cause immediate effects, however due to biomagnification; much of this potential is with the upper trophic organisms. Concentrations of Hg found in the lower trophic organisms, such as the benthic invertebrate *Procambarus clarkii* (red swamp crayfish) often remain at low levels for the entire lifespan. Detrimental effects due to such an exposure remain largely unknown. Benthic invertebrates are at the base of many food webs and therefore could also serve as a potential vector in the Hg flux to higher trophic levels. This along with their natural tendency to have low level Hg exposure makes the *Procambarus clarkii* a model organism to study chronic Hg accumulation, its potential sub-lethal effects, and how this could influence Hg biomagnification.

EXPOSURE AND EFFECTS OF MERCURY

Sources and History of Use

Mercury (Hg) released into the environment can originate from natural and anthropogenic sources, directly or indirectly. Direct contamination of an environment through waste release or spills creates obvious implications for concerns. However, indirect contamination from global Hg emissions may also warrant concern [1, 2]. Until relatively recent times, volcanic and other geothermal activities were the major contributor to global mercury emissions. During the last several centuries the widespread use of mercury in industrial applications, waste incineration, along with the burning of fossil fuels for energy has made anthropogenic activities the major contributor to global emissions [3]. Waste incineration and the burning of fossil fuels currently produce 85% of the total global emissions [3]. Combustion of these products produces Hg vapor and particulates that enter the atmosphere; which may remain there up to a year [3]. Wet (precipitation or snow) or dry deposition usually occurs as the inorganic form (Hg II). Once the Hg II becomes incorporated into aquatic bodies, bacterial conversion of the Hg II to the more toxic methylmercury (MMHg) occurs [4]. The efficiency of this methylation can be influenced by factors such as low pH, high DOC (dissolved organic carbon), frequent flooding/drying, and low oxygen concentrations that are especially prevalent within aquatic environments of the southeastern United States [5-7]. The methylation also contributes significantly to the bioaccumulation and biomagnification of Hg within aquatic systems. [8]

Exposure and Uptake

Exposure of aquatic biota to mercury may occur through diet, water, and sediment. Exposure and accumulation of total (THg) mercury occurs as Hg II or MMHg at different

proportions among trophic levels. While MMHg readily bioaccumulates and biomagnifies, Hg II only bioaccumulates. The diet is likely the most important route of Hg exposure to crayfish in natural environments, and laboratory exposures have shown efficient accumulation with very slow depuration rates of both total Hg (THg) and MMHg [9, 10]. To understand the role of the crayfish in mediating total Hg or MMHg fluxes into food webs, information on accumulation and elimination is required. The diet of crayfish in the natural environment is highly variable and influenced by multiple factors [11, 12]. Crayfish are opportunistic omnivores, feeding on algae, detritus, and animal matter. The contribution of each of these components to the diet varies with availability, and also with the age and sex of the crayfish [11]. Juvenile crayfish prefer a higher proportion of animal protein, whereas adult diets contain higher proportions of algae and moss [11]. Sex differences play a role when food with high nutritional value becomes scarce, because males are typically much larger than females and therefore dominate more nutritional resources during food scarcity [11]. Variability of the crayfish diet could contribute significantly to variation in Hg exposure, along with variation in the proportion of MMHg in the diet.

Given their habitat and dietary habits, crayfish in the natural environment have the potential to be exposed to relatively low concentrations of Hg in their diet during their entire lifespan, with a majority (typically > 50%) of the Hg as Hg II [13-18]. However, juvenile crayfish prefer animal protein, such as fish [11], which would expose them to higher concentrations and proportions of MMHg than a detritus diet. For this reason, accumulation of Hg by juveniles may be a key factor affecting tissue burdens and eventual trophic transfer of Hg to predators. Also, this juvenile diet would suggest relatively higher Hg exposure during early life that could be detrimental to developing organisms.

Effects

Trophic transfer of the Hg burden is only part of the problem of being exposed to dietary Hg. Biota living in wetlands may be exposed to relatively low Hg concentrations in food, water and sediment during their entire lifespan. The potential consequences of chronic dietary exposure are poorly understood in benthic invertebrates. Because MMHg is a potent neurotoxin, exposure may cause adverse behavioral or physiological effects at concentrations far below exposure levels that cause acute lethality [4]. Research to date on exposure of aquatic invertebrates to methylmercury has focused on alterations in limb regeneration [19, 20], molting [19-22], hepatopancreas function [23, 24], and ovarian maturation [25]. Additionally, most studies only have examined exposure to dissolved methylmercury in water. Weis *et al*., studied limb regeneration in three species of fiddler crabs held in water containing 0.1-0.5 mg/L of methylmercuric chloride [19]. Responses ranged from inhibition of melanogenesis to inhibition of limb regeneration, with higher susceptibility at lower salinities. Torreblanca *et al*., exposed crayfish (*Procambarus clarkii)* to 0.25 mg/L mercuric chloride in water, and found that mercury inhibited the replenishment of energetic reserves in crayfish hepatopancreas after starvation [24]. Reddy *et al*., injected 0.5 μg Hg (as mercuric chloride) per gram body weight into *P. clarkii* to examine responses of ovarian maturation [25]. The crayfish that were injected with the mercuric chloride had significantly reduced sizes of mature ovaries and delayed development of maturation. However, the more ecologically relevant dietary exposure pathway, and the potential effects of chronic exposure to Hg via this route on health and behavior have been poorly studied.

CRAYFISH GENERAL BIOLOGY AND BACKGROUND

Systematics and General Description

There are over 540 recognized species of crayfish occurring worldwide. Crayfish exhibit circumtemperate distribution and are native to every continent except Africa and Antarctica [11]. However, the distribution of crayfish fauna is far from uniform, with 77% of the world's crayfish species and subspecies occurring in North America, 20% occurring in Australia, 1.5 % in South America, and 1.5 % in Europe/Asia [11]. The fauna currently consists of three families (Astacidae, Cambaridae, and Parastacidae) and 29 genera; the Cambaridae and Astacidae occur in the North America. The family Astacidae has only one genus, *Pacifastacus*, in North America. This genus occurs on the Pacific slope of the western Unites States and the extreme southwestern Canada. The family Cambaridae, with twelve genera, is the most diverse in the world, and 99% of its members are found in North America [11]. Within the Cambaridae, the genus *Procambarus* contains over 300 species [11]. The crayfish used in the present study, *Procambarus clarkii*, belongs to the family Cambaridae.

The genus *Procambarus:* General Life History, Growth and Reproduction

P*rocambarus* occupy various types of habitat including subterranean burrows, wet meadows, seasonally flooded swamps and marshes, and permanent lakes and streams. *P. clarkii* use burrows during low water, dry periods and/or during reproductive periods. These periods of low water usually coincide with hot months in the lower latitudes, and therefore *P. clarkii* are generally more active in the cool months in these areas. *P. clarkii* burrows are fairly simple, consisting of a sub-vertical passageway that descends in an irregular spiral toward the water table, with rarely more than one opening to the surface. A short, simple chimney of earthen pellets may or may not be present.

Crayfish interpret environmental signals either singly or in concert to determine when conditions are favorable for general activities such as molting and reproduction. Seasonal changes in day length, food availability and temperature are just some of the factors that could influence crayfish behaviors.

At least 11 moults are required before *P. clarkii* are sexually mature. The period between these moults are temperature dependent, with lengths ranging from 6-30 days. Sexually active *P. clarkii* mate whenever they encounter receptive partners [11]. In warmer climates, development is rapid enough to allow at least two generations per year. Once the reproductive activities cease, males moult into a sexually inactive growth phase that closely resembles immature animals. After one or two moults in this stage, the males then revert back to the sexually active form. The females sexual and/or growth phases are not clearly understood. In laboratory conditions, maximum life spans are about 4 years, with a typical life span of 12-18 months.

P. clarkii are generalist and opportunistic when feeding, and cannibalism is common. Microbial-enriched plant debris and detritus is the main source of energy within the diet of an adult. Animal prey is of crucial importance to new hatchlings [26] and is actively consumed by all crayfish when readily available.

Commercial Importance

Procambarus spp., including *P. clarkii*, are harvested primarily in south Louisiana, USA, where much of the region is inundated with superior crayfish habitat [11]. Reported harvest may exceed 30 000 tonnes in a high water year, making the business of significant economic importance [11]. *P. clarkii* is usually cultivated along with rice crops to maximize land use. Open, shallow, earthen ponds are the preferred habitat for cultivation, and ponds are usually drained during the summer months, making the crayfish burrow and become dormant. The

ponds are re-flooded in the late autumn, where the crayfish emerge and are harvested continually through the next spring.

SUMMARY

P. clarkii are most abundant in seasonally flooded wetlands where annual dewatering serves at least three functions: promotion of growth of annual vegetation that provides cover and food when the areas are re-flooded, destruction of many aquatic predators and reduction in anaerobic sediments [11]. Recent studies have shown that the periodic flooding and drying of wetlands that receive atmospheric deposition of mercury maybe associated with substantial bioaccumulation within the associated biota [1-2, 27-29]. Atmospheric deposition of mercury (Hg) has resulted in elevated mercury concentration in biota in many remote and otherwise pristine wetlands due to their high methylation rates [4, 30-33]. The continued exposure to mercury, even at relatively low concentrations, allows for bioaccumulation of mercury because elimination is very slow relative to the rate of uptake [27, 29]. Studies suggest that crayfish often serve as both consumers and prey within aquatic food webs, and subsequently serve as important transformers of energy [11]. Crayfish forage at several different trophic levels within these systems, and with the biomagnification and toxicity of MMHg [4], crayfish of different ages and sex could therefore be exposed to a variety of doses. However, given the preferential dietary habits such as fish by the juvenile crayfish, they have the potential to be exposed to a higher proportion of MMHg in their diet during a critical period of development. For this reason, accumulation of Hg by juveniles may be a key factor affecting tissue burdens, its sub-lethal effects and eventual trophic transfer of Hg to predators.

OJECTIVES OF THE RESEARCH

The red swamp crayfish, *Procambarus clarkii*, has been included in many toxicological studies, but few have categorized how sex and age affect the accumulation, elimination, or potential adverse effects of chronic dietary methylmercury exposure. Therefore, this study seeks to understand the potential relationship sex and age may have in uptake, elimination, and potential adverse effects of dietary methylmercury on the red swamp crayfish *Procambarus clarkii*.

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CHAPTER 2

CHRONIC DIETARY METHYLMERCURY EXPOSURE ON THREE JUVENILE STAGES OF THE CRAYFISH *PROCAMBARUS CLARKII*: ACCUMULATION AND ELIMINATION[1]

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[1] Brant, H.A, C.H. Jagoe, A.K. Wall, J.M. Unrine, W.A. Hopkins and P.L. Williams. To be submitted to *Environmental Toxicology and Chemistry*.

CHAPTER 2

CHRONIC DIETARY METHYLMERCURY EXPOSURE ON THREE JUVENILE STAGES OF THE CRAYFISH *PROCAMBARUS CLARKII*: ACCUMULATION AND ELIMINATION

ABSTRACT

Benthic invertebrates such as crayfish serve as a critical intermediary in the movement and biomagnification of mercury (Hg) from lower to upper trophic levels. To understand the role of crayfish in trophic transfer of Hg, information on accumulation and elimination in these organisms is required. This study used a 2x2x3 randomized block design to examine accumulation and elimination of Hg by juvenile crayfish (*Procambarus clarkii*, n=72) of known age and sex chronically exposed to environmentally-realistic dietary Hg concentrations. Artificial diets were made containing an average of 9 ppb Hg fresh weight (80% MMHg) or 278 ppb Hg fresh weight (98% MMHg). There were sex and age related differences in Hg accumulation, but these relationships were not evident in elimination. Molting eliminated relatively little mercury. Hg concentrations were much higher in pre-molt exoskeletons than in molted ones, suggesting remobilization of Hg from the exoskeleton prior to molting.

INTRODUCTION

Mercury (Hg) from atmospheric deposition tends to accumulate in biota inhabiting wetlands that are critical habitats for many species such as crayfish [1]. Inorganic Hg (as Hg II) is the major species deposited in precipitation. Conversion of Hg II to the more toxic methylmercury (MMHg) is enhanced by factors such as low pH, high DOC, and low oxygen concentrations that are especially prevalent within aquatic environments of the southeastern

United States [1-3]; the native range of the Procambarus genus. MMHg readily bioaccumulates in exposed organisms and biomagnifies to high concentrations within food webs [3]. Benthic invertebrates such as the crayfish *Procambarus clarkii* (*P. clarkii*) serve as a critical intermediary in the movement and biomagnification of mercury from lower to upper trophic levels [4] because of their role as a prey item. The proportion of Hg II to MMHg is typically higher at the lower trophic level occupied by crayfish (usually greater than 50%); however, Hg II does not biomagnify [4, 5]. To understand the role of the crayfish in mediating total Hg or MMHg fluxes into food webs, information on accumulation and elimination is required.

The diet is likely the most important route of Hg exposure to crayfish in natural environments, and laboratory exposures have shown efficient accumulation with very slow depuration rates of both total Hg and MMHg [6, 7]. The diet of crayfish in the natural environment is highly variable and influenced by multiple factors [8]. Crayfish are opportunistic omnivores, feeding on algae, detritus, and animal matter. The contribution of each of these components to the diet varies with availability, and also with the age and sex of the crayfish [8]. Juvenile crayfish prefer a higher proportion of animal protein, whereas adult diets contain higher proportions of algae and moss [8]. Sex differences play a role when food with high nutritional value becomes scarce, because males are typically much larger than females and therefore dominate more nutritional resources during food scarcity [8]. Variability of the crayfish diet could contribute significantly to variation in Hg exposure, along with variation in the proportion of MMHg in the diet.

Given their habitat and dietary habits, crayfish in the natural environment have the potential to be exposed to relatively low concentrations of Hg in their diet during their entire lifespan, with a majority (typically > 50%) of the Hg as Hg II [9-13]. However, juvenile crayfish

prefer animal protein, such as fish [8], which would expose them to higher concentrations and proportions of MMHg than a detritus diet. For this reason, accumulation of Hg by juveniles may be a key factor affecting tissue burdens and eventual trophic transfer of Hg to predators. Also, this juvenile diet would suggest relatively higher Hg exposure during early life that could be detrimental to developing organisms. Therefore, this study seeks to understand potential relationships among sex, age, uptake and elimination in red swamp crayfish exposed to chronic dietary methylmercury.

MATERIALS AND METHODS

Preparation of Experimental Diet

Two diets were formulated from a combination of finely ground spirulina (Superpetz, LLC), brine shrimp (Superpetz, LLC), alligator chow (Burris Mill and Feed, LA) and fish fillets, all embedded in a matrix of high quality gelatin and agar (Fisher Scientific). The agar and gelatin were brought to boiling within a liter of water. The spirulina, shrimp and shredded fish fillets were then added to the mixture and gently stirred to homogeneity. The solution was then poured into a 33 X 23 cm container that contained a 2.54 X 2.54 cm polycarbonate grid along the bottom, spread evenly and allowed to cool and solidify at 1^0C refrigeration. The grid provided multiple cubes of food that were homogeneous in size. Each mixture was made as two batches for each diet due to volume limitations.

Mercury concentrations for the two diets were adjusted by the addition of fish fillets from two different sources. Fish for the high Hg diets were wild largemouth bass (*Micropterus salmoides*) from SRS reservoirs collected by angling. Pervious work has shown that these fish contain elevated concentrations of mercury [14]. Fish for the low Hg group were commercially available farm raised catfish (*Ictalurus punctatus*) purchased at a local grocery store. The low

Hg diet contained a mean concentration of 0.009 μg/g fresh weight (80% MMHg), while the high Hg diet contained a mean concentration of 0.278 μg/g fresh weight (98% MMHg). The low diet was not labeled "control" because of the small amount of detectable Hg, but probably represents the lowest mercury concentration that could reasonably be achieved in a diet containing uncontaminated freshwater fish. Neither diet contained sufficient concentrations of MMHg to be acutely toxic, and the range of concentrations are within those that are currently found in aquatic systems in the southeastern US [1, 2].

Samples of both diets were lyophilized to measure moisture content. There were no differences between the high Hg diet (mean = 73% moisture) and the low Hg diet (mean = 74% moisture). Mean weights ($\pm$ standard error) of the cubes were also the same for each diet, 1.77 $\pm$ 0.22 grams and 1.81 $\pm$ 0.17 grams for the high Hg and low Hg diets, respectively.

Nutrient Analysis of Diet

Proximate analysis of the diet was performed in triplicate (Table 2.1). Crude fat content was determined as the loss in mass after hot petroleum ether extraction using a semi-automated extraction system. Ash content was determined as the residue remaining after burning a sample at 550^{o} C. Energy, crude protein, and crude fiber determinations were made by the University of Georgia, Department of Poultry Science Analytical Research Services Laboratory. They determined energy content by bomb calorimetry and crude protein content using the Dumas combustion method (combustion, gas chromatography-thermal conductivity). They determined crude fiber by calculating the loss of mass on ignition from dry samples rendered free of fat by ether extraction and digested in weak acid followed by weak base.

Experimental Procedure

Mature adult crayfish purchased from a local farmer were kept within a large (4.6 X 14.6 m) concrete room containing slowly-flowing well-water (20 L/hr) at a depth of approximately 20 cm, with multiple hiding places created by concrete blocks. Well water pH was approximately 6.6 and conductivity was approximately 110 μS/cm. This water was used during acclimation, breeding and the feeding experiments. Crayfish were allowed to breed, and upon observation of females bearing eggs, they were removed from the general population. To assess any maternal contribution of Hg to the young, 10 randomly chosen female crayfish were euthanized and analyzed for total Hg. Mean mercury concentration ($\pm$ standard error) in these females was 32.16 $\pm$ 7.81 ng /g dry weight, suggesting very low potential for maternal transfer. The "in berry" females (those with eggs) were placed within in one of two (213 L x 70 W x 56 D cm and 274 L x 70 W x 56 D cm) Living Stream Systems tanks (Frigid Units, Inc., Toledo, Ohio), which were evenly divided into five compartments. Well water (10 L/hr) flowed through the tanks, and depth was keep at approximately 70 cm so that the females could crawl up and on the dividers creating the compartments within the streams. Females within each compartment were divided according to the date when eggs were observed. This was used to categorize ages when the juveniles were ready to be removed from the female. The juveniles were categorized into three distinct age classes, all within 3-4 weeks of each other. Age one, the youngest, were approximately three weeks old at the beginning of the feeding experiment and had reached the fourth molt. Age two, the middle age group, were approximately five weeks old at the beginning of the experiment and had reached the sixth molt. Age three, the oldest age group, were approximately eight weeks old when the feeding experiment began and had reached the eighth

molt. Crayfish are considered adults after 12 molts, therefore the age 3 juveniles were considered slightly immature.

The experiment used a 2X2X3 randomized block design (2 diet treatments, 2 sexes, 3 age classes) with a total of 72 crayfish. Figure 2.1 shows the assignments of each Hg treatment, age and sex. Crayfish were randomly assigned a diet containing either a low or high Hg concentration and were each housed within an individual 946mL polyethylene container modified with three screened sides, vented bottoms (for waste removal), and fitted with foam rings for floatation. These containers were placed in one of two (213 L x 70 W x 56 D cm and 274 L x 70 W x 56 D cm) Living Stream Systems tanks (Frigid Units, Inc., Toledo, Ohio), which were each evenly divided into two compartments. Each compartment was assigned high or low Hg diets. Tanks received continuous, flowing well-water with aeration at a rate of about 10 liters/hour. Water temperature ranged from 16 to 18 ^{0}C. Crayfish were fed ad lib, and checked for molting daily. A cube of diet was placed in each container with an individual crayfish, and renewed when it had been nearly consumed. Growth was monitored by weight measurements (grams) every seven days. Crayfish were euthanized and dissected at 142 days. Tissues (abdominal muscle, abdominal exoskeleton, ovaries if present, gastroliths if present, and the digestive gland) were collected and lyophilized to constant weight.

Analyses - Total Mercury

Tissues (about 30 mg/sample) and diets (about 75 mg/sample) were analyzed for total mercury following EPA method 7473, using a DMA80 Direct Mercury Analyzer (Milestone, Inc, Monroe, CT, USA). This method utilizes thermal decomposition, gold amalgamation, thermal desorption and CVAA detection. Samples were analyzed in batches of ten, with each batch including a blank, a sample replicate, and a tissue standard certified for Hg concentration

(DORM-2, dogfish muscle, or TORT-2, lobster hepatopancreas, purchased from the National Research Council of Canada (NRCC), Ottawa, Canada or BCR-60, aquatic plant, *Lagarosiphon major*, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium). Based on a 30 mg or 70 mg sample and an average blank of 0.1688 ng Hg (n=25), the method detection limit (MDL) was 5.63 ng /g and 2.25 ng/g, respectively. The average tissue standard recovery was 96% (n=38). The average difference between sample replicates was 5% (n=29).

To address possible matrix interferences, some tissue was analyzed for total mercury after chemical digestion in batches, with microwave assistance following EPA method 3052. Each batch included a sample replicate, a reagent blank and a certified tissue reference material (Tort-2 (lobster hepatopancreas) NRCC, Ottawa, Canada or BCR-60, aquatic plant, *Lagarosiphon major*, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium). Briefly, approximately 0.1g of sample was weighed into a 120 ml Teflon vessel, 5mL of trace metal grade nitric acid (HNO_3) was added and samples were heated in a MDS-2000 laboratory microwave (CEM Corp., Mathews, NC). The procedure involved heating steps of 50, 60, 70, and 80% microwave power for 10, 10, 15, and 20 minutes, respectively. Upon completion of the digestion, samples were brought to a total volume of 25mL using double de-ionized H_20 containing 1% BrCl (Hg preservative). Mercury analysis was performed by cold vapor atomic fluorescence spectroscopy (CVAFS, Brooks-Rand Ltd.). Calibration standards covering a range of 1-10µg/L were prepared by serial dilution of NIST traceable primary standards. Based on a 100 mg sample and an average blank of 0.01 ng Hg (n=11), the method detection limit (MDL) was 2.50 ng/g. The average tissue standard recovery was 95% (n=12). The average difference between sample replicates was 8% (n=25). The average spike recovery was 94% (n=8).

Analyses - Methyl Mercury

Briefly, approximately 75 mg of sample was weighed into heavy walled acid washed polytetrafluoroethylene (PTFE) vials containing 5 ml of 25% (w/v) KOH in methanol and allowed to digest at 70 ^{o}C overnight. Digestates were then diluted to 10 ml with methanol. Small aliquots of the digestates were directly analyzed for inorganic mercury (Hg II) and methylmercury (MMHg) by aqueous phase ethylation, room temperature pre-collection on Tenax, with detection by isothermal gas chromatography and cold vapor atomic fluorescence spectrophotometry (GC-CVAFS) (Brooks Rand Model III atomic fluorescence spectrophotometer, Brooks Rand, Seattle, WA, USA) using the methods of Liang *et al*. [15]. Methylmercury and Hg (II) concentrations were determined by direct standardization. Peaks for other Hg species were not present in the chromatographs indicating that the sum of the MMHg and Hg (II) concentrations was approximately equivalent to the THg concentration in our samples. A National Institute of Standards and Technology (NIST) traceable standard was used for Hg (II) and the actual titer of the MMHg standard was determined as described in United States Environmental Protection Agency (EPA) method 1630 (EPA, 2001). Each analytical batch of 24 samples included two extraction blank samples, two standard reference material samples (TORT-2 lobster hepatopancreas, National Research Council of Canada (NRCC), Ottawa, ON, Canada and BCR-60, aquatic plant, *Lagarosiphon major*, Institute for Reference Materials and Measurements (IRMM), Geel, Belgium), and one sub-sample replicate. Analytical spike recovery was determined in duplicate for each Hg species for each analytical batch as well as relative percent difference (RPD) between replicate analyses. Detection limits for a 50 mg sample based on the moisture contents of the diets being 73 % and 74 % were 2.52 ng Hg/g fresh wt. and 2.25 ng Hg/g fresh wt. for Hg II (Table 2.2). Detection limits for a 50 mg sample based

on the moisture contents of the diets being 73 % and 74 %were 1.96 ng Hg/g fresh wt. and 1.75 ng Hg/g fresh wt. for MMHg (Table 2.2). Concentrations in most samples exceeded these detection limits. Those below the detection limits (n=3) were assigned a value of half of the detection limit for statistical analyses. Recovery of MMHg and THg from TORT-2 was 98% and 99% respectively (tort-2 is not certified for Hg II content). Analytical spike recovery was 106% for MMHg and 116% for Hg II. Relative percent difference between replicate analyses was 10% for MMHg and 11% for Hg II.

Analyses - Other Metals

Sub-samples of each diet (high and low Hg) were digested according to EPA method 3052 using microwave heating as discussed previously. Samples were analyzed for Al, Cr, Fe, Ni, Cu, Zn, As, Se, and Pb according to EPA method 6020, using an ICP-MS Elan DRC Plus, (Perkin-Elmer Sciex Instruments, Toronto, Canada). For every 28 digested samples (which included replicates, blanks, and standard reference materials as mentioned previously), there were dilution replicates, spiked samples, and a standard addition series to correct for potential matrix interferences. Table 2.3 lists all major quality control and quality assurance parameters.

Statistical Analysis

All statistics were performed using SAS for Windows (version 9.0; Cary, NC, USA). Potential outliers in the data were identified by Grubbs' test, which was also used to determine if such outliers should be excluded from the data set. Two outliers were removed from the data set; one age three female from each Hg diet treatment. Data were checked for normality and homoscedasticity required for parametric analysis using Shapiro-Wilk's and Bartlett's tests respectively. A three-way ANOVA with block effects was used to determine differences between concentrations in tissues. Nonparametric analyses were preformed on nutrient and

metal analysis and mortality data (proc NPAR1WAY WILCOXON). All calculations are expressed on a dry weight basis.

RESULTS

Table 2.1 shows the nutrient analysis of the experimental diets, including energy, crude protein, fiber, ash, and lipid. Crude protein, fiber, and lipid are expressed as percent of dry weight. Energy is expressed as calories per gram. All values are means of three replicate analyses. This analysis revealed a difference in lipid ($p=<0.00001$, $F_{1, 4}=4521.05$), protein ($p=<0.0495$, $\chi^2=3.86$), fiber ($p=<0.0495$, $\chi^2=3.86$) and energy ($p=<0.0495$, $\chi^2=3.86$) content between the two diets (Table 2.1). The high Hg diet contained 15% lipid, while the low Hg diet contained only 5% lipid. The high Hg diet contained 5229.50 cal/g and the low Hg diet contained 5678.23 cal/g. There is also a difference between the percent protein. The diets yielded 66% and 58% for the high and low Hg treatments, respectively. Fiber content was 0.51 for the low Hg diet and 0.71 for the high Hg diet.

Table 2.2 includes the MMHg analysis of the two experimental diets. Values are listed as methylmercury (MMHg), inorganic mercury (Hg II), with both of these combined for total mercury, and the percentage of this total that is methylmercury listed in the last column. Values are expressed in ng/g on a fresh weight basis. Below detection limit is listed as “BD”, with one half of this value assigned. There was siginificant differences between treatments for MMHg ($p=0.0495$, $\chi^2=3.86$) and THg ($p<0.0001$, $\chi^2=24.77$), but not Hg II. MMHg analysis of the high Hg diet yielded an average of 240 ng/g THg, with 99% as MMHg (Table 2.2). MMHg analysis of the low Hg diet yielded an average of 9 ng/g THg, with 79% as MMHg (Table 2.2).

Table 2.3 includes the quality control and quality assurance parameters of the metal analyses for the two diets. Digestion replicate difference is the percent difference between one

sample that was digested twice. Dilution replicate difference is the percent difference between one sample that was diluted twice. Standard refernce material recovery is the percent recovery of the certified published value. Spike recovery is the percent recovery of a spike of a sample with a known value of the element. The method detection limit could not be calculated for this analytical batch because only one digestion or reagent blank was analyzed. However, other analyses of similar materials by this lab indicated that method detection limits were sufficiently low to adress the question of whether any other elements were present at elevated or potentially toxic concentrations. Values of the digestion or reagent blank are listed as ppm. All of these parameters were within expected guidelines, except for the somewhat elevated recovery of selenium (144%) in the standard reference material. Metal analysis (Table 2.4) indicated differences only in Se ($p=<0.009$, $\chi^2=6.82$) and Ni ($p=<0.0361$, $\chi^2=4.39$) concentrations (other than mercury) between the two diets. However, these differences were thought to be negligible given the very low concentrations of these trace elements [11, 16-21]. Table 2.4 values are means of five replicate analyses, and are expressed as μg/g on a fresh weight basis.

Eleven crayfish of the 72 total died during the experiment: 2 in the low Hg diet treatment and 9 in the high Hg diet treatment (Table 2.5). Because they had been exposed to dietary Hg for a shorter period than those that survived the entire 142 days, those that died early were not included in the growth and mercury concentration analyses. However, Table 2.5 lists the experimental day and multiple tissue concentrations for each mortality. The two mortalities within the low Hg treatment were both males from the middle age group (age 2). The nine mortalities of the high Hg treatment consisted of one male and one females of the youngest age group (age 1) and three males and four females from the middle age group (age 2). There were

no deaths in the oldest age group (age 3) for either Hg treatment. Mortality differed significantly between the Hg diet treatments ($p=0.025$, $\chi^2=5.25$).

Figure 2 shows the mean Hg accumulation in four tissue types by Hg exposure, sex and age. The values are expressed as THg (ng/g dry weight) with each bar representing the mean $\pm$ one standard error. Mean Hg concentration in abdominal muscle, digestive gland, exoskeleton, and ovaries (Figure 2.2) all varied significantly between Hg treatments (muscle: $p<0.0001$, $F_{11,47}=598.75$; dig. gland: $p<0.0001$, $F_{11,47}=130.74$; exoskeleton: $p<0.0001$, $F_{11,47}=153.28$; ovaries: $p<0.0001$, $F_{11,47}=182.48$). The crayfish fed the high diet accumulated a mean Hg concentration that was orders of magnitude above the crayfish fed the low Hg diet. Mean Hg concentration in abdominal muscle, exoskeleton and ovaries (Figure 2.2) also varied significantly among ages (muscle: $p=0.0369$, $F_{11,47}=3.54$; exoskeleton: $p=0.0007$, $F_{11,47}=8.50$; ovaries: $p=0.0123$, $F_{11,47}=5.41$). The general trend for both Hg diet groups indicates that the older crayfish tended to accumulate less Hg than the younger crayfish. There was also a significant interaction (Figure 2.2) between age and Hg treatment in abdominal exoskeleton ($p=0.0031$, $F_{11,47}=6.53$) and ovaries ($p=0.0054$, $F_{11,47}=6.69$), and a marginally significant interaction for abdominal muscle ($p=0.0591$, $F_{11,47}=3.01$). This indicates Hg accumulation in these tissues varied among ages within and between treatments. For instance, the older females in the high Hg treatment tended to have less Hg in the ovaries while the older females in the low Hg treatment showed the opposite trend. Mean Hg concentrations for the abdominal muscle was also significantly different between sexes within and between Hg treatments (($p=0.0469$, $F_{11,47}=4.17$) (Figure 2.2). The females tended to accumulate more Hg than the males fed the high Hg diet, whereas the males tended to accumulate more Hg than the females fed the low Hg diet. There was also an interaction between the sex and Hg treatment (($p=0.0369$, $F_{11,47}=3.54$). The

exception to the trend listed previously is the difference between the males and females fed the low Hg diet in the oldest age group. The mean Hg concentration in males was lower than that in females in this group.

Gastroliths are small calcified paired bodies that are formed between the epidermis and foregut during pre-molt. Gastrolith Hg accumulation was not included in Figure 2.2 due to the small number of crayfish from each group that contained them. Gastrolith Hg concentrations for the high and low crayfish ranged from 0.06-749.28 ng Hg/g dry wt. (n=13) and 0.055-0.06 ng Hg/g dry wt. (n=3), respectively, with a mean and one standard error of 163.69 $\pm$ 66.21 and 0.06 $\pm$ 0.001 ng Hg/g dry wt, respectively. Due to the small sample size, statistical analysis was not performed.

Tissue concentration patterns varied between the two diets (Figure 2.2). The crayfish fed the high Hg diet accumulated the most Hg within the abdominal muscle>digestive gland>abdominal exoskeleton> ovaries> gastroliths >molts. The crayfish fed the low Hg diet accumulated Hg in a different pattern: abdominal muscle>abdominal exoskeleton>digestive gland>ovaries>molts>gastroliths.

Figures 2.3, 2.4 and 2.5 show the mean Hg concentrations in exoskeletons and in molted exoskeletons by exposure and age. These data were calculated by averaging Hg concentrations for each age and treatment group for pre-molted exoskeletons and post molt exoskeletons. Sex differences were not significant, so sexes were pooled. Pre-molted exoskeleton is defined as the abdominal exoskeleton that was part of each crayfish at the end of the experiment (day 142). The assumption is that this represents the Hg concentration that would be found within the exoskeleton prior to molting. Post-molt exoskeleton (molt 1-4) is defined as the abdominal exoskeleton that was shed away from the body during earlier parts of the experiment. Each bar

within the figures represents the mean ± one standard error. The number of individuals represented in each bar is included along the bottom, and the mean day for each molt is listed above the bar. The percentage of Hg shed in the molted exoskeleton as compared to the exoskeleton prior to molt is also listed above each bar. This percentage might be an underestimate due to the fact that the amount of Hg that any particular crayfish contained was much higher at the end as compared to the beginning of the experiment. However, the trends shown are still indicative of the behavior of the Hg shed into the molted exoskeleton. Regardless of age or Hg exposure, Hg in pre-molt exoskeleton greatly exceeded Hg in the molted exoskeleton (Figures 2.3-2.5). This suggests mobilization and resorption of mercury from the exoskeleton prior to molting.

Even though the mean Hg concentration in pre-molt exoskeletons was significantly different among ages within the Hg treatments ($p=<0.0007$, $F_{11, 47}=8.50$), elimination of the body burden into the molted exoskeleton did not vary significantly by age. Statistical analysis revealed that the Hg concentration of molted exoskeletons only differed between the diet treatments for molts two thru four (molt 2: $p=0.0068$, $F_{4, 42}=8.11$; molt 3: $p=0.0031$, $F_{3, 27}=10.59$; molt 4: $p=0.0004$, $F_{3, 9}=29.10$); neither age nor sex was significant. The molts were significantly different from the pre-molt exoskeleton within each Hg treatment ($p=<0.0001$, $F_{12, 189}=24.13$), however the molts within each Hg exposure where not different from one another (Figure 2.3-2.5).

Regardless of age, Figures 2.3-2.5 indicate that most Hg was resorbed from the exoskeleton before molt. In the crayfish fed the high Hg diet, an average of only 3 % of the mercury in the exoskeleton was present after the exoskeleton was molted. In the crayfish fed the

low Hg diet, an average of 15% of the concentration present in the exoskeleton was found in the molted shell.

DISCUSSION

Nutrient and metal analyses of the experimental diets were conducted to ensure the two diets were similar in composition. Several differences were found that warrant discussion. There were differences between the two diets in fiber, lipid, protein and energy content (Table 2.1). The low Hg diet contained 15% lipid, while the high Hg diet contained 5% lipid. The low Hg diet had 58% protein, while the high Hg diet had 66% protein. The low Hg diet consisted of 5678 cal/g energy (5.7 Mcal/kg), while the high Hg diet consisted of 5230 cal/g energy (5.2 Mcal/kg). The low Hg diet fiber was 0.55 while to high Hg diet was 0.71.

It is possible that these observed differences could affect uptake and elimination patterns. However, previous work suggests that the magnitude and direction of these differences did not compromise the results. Jover *et al*. [22] concluded that optimum nutrient requirements for *P. clarkii* juvenile growth to be around 30% protein, 2.4-2.9 Mcal/g of energy content, with a lipid content of 6%. The also found that higher lipid concentrations (13% and 11%) at a protein level of 25-30% reduced growth and survival. Huner and Meyers [23] also found reduced growth with 20-25 % protein and high lipid content (19 % and 17%) as also reflected in other literature [24, 25]. However, Ackerfors *et al*. [25] found that the increase in lipid from 5.5% to 10% at a protein content of 40% did not reduce growth, nor increase mortality. The diets formulated for this experiment contain energy (5.3 and 5.7 Mcal/kg) and protein (58% and 66%) levels well above such published values. Thus, it is unlikely that the protein or caloric contents of the diet restricted growth and development. The lipid value differences of 5% in the high Hg diet and 15% in the low Hg diet are also above minimum requirements, with some evidence, as outlined

above, that a lipid content of difference of 10 % between the two diets might be negligible due to the high protein and energy values. Published values of dietary fiber for crustaceans were not located and therefore the effect of this difference between the two diets remains unknown.

Since fish used were from different locations, with the potential for exposure to other pollutants, it was necessary to verify that the effects observed were due to the Hg concentrations and not by another toxic metal. Metal analysis of the experimental diets shown in Table 2.4 indicates differences in the Se and Ni concentrations between the two diets. Review of published literature for values within food items and bodily organs indicates that the differences between the concentrations are minimal and the concentrations are thought not to be acutely toxic [11, 16-21].

Chronic exposure to the high Hg diet resulted in higher mortality than the low Hg diet (Table 2.5). Nine of the eleven crayfish that had early mortality were in the high Hg diet treatment. The two crayfish that died in the low Hg diet treatment were both males from the middle age group (age 2). There were no deaths in the oldest age group (age 3) for either diet treatment. This suggests that juveniles are more sensitive to chronic exposure to environmentally realistic Hg concentrations than older individuals. Patterns did not exist in the amount of time until mortality within or between groups. The Hg concentration for each individual that died is difficult to compare to the individuals that survived until the termination of the experiment because of the difference in period of dietary exposure.

This research suggests that age and sex influence uptake and elimination when juvenile crayfish are fed ecologically-realistic dietary concentrations of Hg. Mean Hg concentration in abdominal muscle, digestive gland, exoskeleton, and ovaries (Figure 2.2) all varied significantly between Hg treatments, and the crayfish fed the high diet accumulated mean Hg concentrations

that were orders of magnitude above the crayfish fed the low Hg diet. Accumulation of mercury in tissues differed depending on age, sex and Hg exposure (Figure 2.2), and accumulation patterns varied somewhat between the two diets (Figure 2.2). Regardless of diet, crayfish accumulated the highest Hg concentrations in their abdominal muscle. However, there were differences between the diets in those tissues that accumulated the next highest Hg concentrations. The crayfish fed the low Hg diet accumulated more of the body burden within the exoskeleton, while those fed the high Hg diet accumulated more of the body burden within the digestive gland. Both sets of crayfish may be physiologically adjusting to the body burdens in different ways. The crayfish fed the high Hg diet could be experiencing sub-lethal stresses due to the metal possibly overtaxing repair or compensation mechanisms, and therefore could be partitioning the Hg burden in a different manner than the crayfish fed the low Hg diet. The relatively higher accumulation in the digestive gland relative to the exoskeleton with the high Hg diet suggests that the exoskeleton is less important as a potential excretion route with higher Hg exposure, and that Hg accumulating in the digestive gland might begin to interfere with normal physiological functions, although the concentration that would cause harmful effects remains unknown.

Comparison of the observed trends within crayfish tissues to other literature becomes difficult due to differences in exposure routes and ages. Simon *et al*. [6] concluded that exposing 1 year old male *Astacus astacus* crayfish (presumed to be adult) to clam tissue containing a mean of 3370 $\pm$ 79 ng Hg/g (FW) of MMHg for 15 days resulted in no differences between muscle and hepatopancreas concentrations, with the highest concentrations accumulating within the green gland. Mason *et al*. [11] concluded that as MMHg became more bioavailable, Hg tissue concentrations are highest in the muscle followed by the gonad. These results were from feral

crayfish from two different rivers where neither the species of crayfish, nor the sex and age for these particular Hg determinations were listed.

Mean Hg concentration in abdominal muscle, exoskeleton and ovaries (Figure 2.2) varied significantly among ages. The general trend for both Hg diet groups indicates that the older crayfish tended to accumulate less Hg than the younger crayfish. This could be the result of dilution by increasing body mass (growth dilution). Mason *et al*. [11] found that as crayfish age increased (indicated by size), MMHg concentration also increased. It is important to remember that, in the present study, three age groups were simultaneously fed high or low mercury diets. While accumulation varies among ages, it does not necessarily follow that older crayfish will contain less Hg than younger crayfish. Instead, our results show that the rate of accumulation slows as crayfish age. Our results are consistent with the idea that, in a natural population, older crayfish will have accumulated higher Hg concentrations than younger individuals.

Although there were differences in tissue concentration between diets, Hg accumulated within ovaries in both treatments. This provides evidence that when exposed to Hg, crayfish can accumulate Hg within the ovary and therefore potential for maternal transfer exists. This raises concern about potential effects on developing oocytes. In the present case, females bred to provide juvenile crayfish for this experiment had low mercury concentrations, so the potential for maternal transfer was small. In other environments, the amount of Hg transferred into eggs could be greater and the potential effects on developing embryos more severe. Mason *et al*. [11] concluded similar findings. Feral crayfish that were exposed to MMHg accumulated a substantial proportion of their body burden within the gonads.

Abdominal muscle was the only tissue that showed a statistically significant difference in Hg concentration between sexes with Hg treatments (Figure 2.2). The females tended to

accumulate more Hg than the males fed the high Hg diet. While there is no literature on sexual differences in crayfish that might explain this observation, Goulet *et al*. [27] noted differences between sexes in response to Hg in mammals. They chronically exposed fetal and early postnatal male and female mice to methylmercury and found that the females performed more poorly than the males in a majority of behavior trials. They concluded that sex-dependent behavior responses could be a result of the females' lower capacity to maintain glutathione levels (GSH) in the brain after treatment with MMHg. If GSH levels are lower in adult females than in adult males, females could eliminate MMHg from liver to circulation less efficiently than males [27]. If this trend extends to crayfish, females fed the high Hg diet could be accumulating more Hg in their muscle tissue because of the less efficient transport to and elimination by the digestive gland. Crayfish fed the low Hg diet exhibited just the opposite trend; males tended to accumulate more Hg within the abdominal muscle than the females, except in the oldest age group. Although the difference was not statistically significant, the mean Hg concentrations within the digestive glands of males fed the low high diet was higher than the females (Figure 2.2). This supports the concept that the females may be less efficient in circulating the Hg to the digestive gland, possibly due to reduced GSH levels.

The abdominal exoskeleton Hg concentrations showed significant differences among exposures and ages, but not sex (Figure 2.2). Concentrations were similar in ages one and two, but lower in age three, the oldest individuals, fed the low Hg treatment. The crayfish fed the high Hg diet displayed significantly difference Hg concentrations in exoskeletons among ages, with the lowest concentrations in the oldest cohort. Crustaceans must shed their exoskeleton as a function of growth and therefore the potential exists for eliminating xenobiotics via the shed exoskeleton. Therefore, mean Hg accumulation within each shed exoskeleton (a molt) was

determined and compared to the exoskeleton prior to molt as seen in figures 2.3-2.4. Exoskeleton dissected from the crayfish upon euthanization at day 142 is considered to be the condition during inter-molt stages. The mean Hg concentration within this pre-molt exoskeleton was found to be significantly different among Hg treatments and ages (Figures 2.3-2.5). The drastic differences detected between the Hg concentrations in pre-molt exoskeleton and the shed or "molted" exoskeleton (Figures 2.3-2.5) for both high and low Hg exposures, suggests mobilization from the exoskeleton and re-absorption of mercury prior to molting. Crayfish exoskeleton is a multi-layered structure comprised of uncalcified and calcified layers. The uncalcified membranous endocuticle contains multiple substances that would be expected to have a high affinity for Hg [28]. Crustacean cuticles contain glycosaminoglycan-sulfur groups (GAG), keratin-like compounds, and cysteine thiols [29-31], all rich in –SH groups that might serve as binding sites for Hg. Any Hg thus bound in the exoskeleton could mobilize when endocuticle is removed by acidic dissolution processes and reabsorbed just before the exoskeleton is shed from the body [29]. Gastroliths are paired calcified bodies that form prior to and during this dissolution process [8]. The gastroliths contain a small amount of an organic matrix that is mostly comprised of chitin and undetermined proteins [32-36]. Therefore during the dissolution process potential exists for the mobilized Hg from the endocuticle to become localized within the gastrolith matrix for re-absorption once the exoskeleton begins to re-mineralize and harden. The Hg concentrations of the high Hg group found in the limited number of gastroliths provide some evidence to support this theory. However, the variation was extremely high (range 0.06-749.28 ng Hg/g dry wt. (n=13)) indicative of two possibilities. The gastroliths could be at different stages of re-absorption at the time of Hg analysis, or the organic

matrix within each individual gastrolith may be highly variable by nature and therefore variable in Hg content.

Even though the mean Hg accumulation within pre-molt exoskeleton was significantly different among ages within the Hg treatments, elimination of the body burden into the molted exoskeleton did not vary significantly by age. Statistical analysis revealed that the concentration of Hg in shed exoskeletons only differed between dietary treatments in molts two thru four, and neither age nor sex was a significant factor. The lack of difference between the ages for the amount of Hg shed into the molt is in contrast to the pattern of higher Hg accumulation with decreasing age in the pre-molt exoskeleton. While the molts were significantly different from the pre-molt exoskeleton, the molts within each Hg exposure where not different from one another (Figure 2.3-2.5). In each case, only a small fraction of the mercury concentration present in the pre-molt exoskeleton occurred in the molted exoskeleton. Moreover, the concentration in the molted exoskeltons was generally a small fraction of the concentrations found in other tissues, including digestive gland and abdominal muscle. These observations strongly support the conclusion that molting is not a significant elimination pathway for Hg in juvenile crayfish.

Crayfish of the high Hg exposure were designed to be a worse case scenario for those juveniles that sustain a greater portion of their diet with animal protein such as fish. This research indicates that if this exposure occurs chronically throughout most of a juvenile life, substantial tissue Hg concentrations result. In fact, the concentrations for this exposure far exceed those concentrations of published values [9-13, 37], including those environments with direct Hg contamination. The substantially lower published values of Hg tissue concentrations indicate that variability in the components of the crayfish diet must be a factor in keeping Hg exposure relatively lower. While crayfish that consume fish might realistically be exposed to 278

ng Hg/g, the lower tissue concentrations reported for wild crayfish suggest that their actual diet contains items with substantially lower hg concentrations. Published tissue Hg concentrations [9, 11-13] from locations with no point source are in the range of 80-200 ng Hg/g dry wt, which compare more closely with the resulting tissue Hg concentrations of the crayfish fed the low Hg diet within this experiment. This indicates, along with previously published literature, that crayfish exposed to modest / realistic concentrations in the environment can accumulate enough Hg to be hazardous to regular consumers [9-13]. This implicates that crayfish serve as important vectors in the flux of THg and MMHg into higher trophic levels with predators such as wading birds, large mouth bass, raccoons, and even humans.

CONCLUSION

There is sex and age related differences in Hg accumulation when crayfish are chronically exposed to environmentally realistic concentrations in the diet. The younger crayfish from the high Hg exposure accumulated more Hg, with higher mortality than the older crayfish of the same exposure. The females of the high Hg exposure had higher Hg tissue concentrations than the males. However, the relationships of sex and age are not evident in elimination, with suppression of Hg elimination through the molted exoskeleton in both Hg exposures. Major differences seen between the pre-molt and molted exoskeleton of both Hg exposures suggests mobilization and re-absorption of Hg. All of these factors could confound understanding the flux of THg and MMHg into higher trophic positions and therefore should be included within any future studies.

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Table 2.1. Nutrition analysis of experimental diets. Values are means of three replicate anaylses with one standard deviation beside each value in paratheses. Crude protein, fiber, ash, and lipid are expressed as percent of dry weight. There was a significant differenence in lipid, protein, fiber and energy content between treatments.

Treatment	Energy (cal/g)	% Crude Protein	% Crude Fiber	Ash %	Lipid %
Low Hg diet	5678.23 (12.77)	58.30 (0.39)	0.55 (0.01)	21.00 (2.65)	15.34 (0.24)
High Hg diet	5229.50 (31.09)	66.01 (0.33)	0.71 (0.01)	18.67 (0.58)	5.33 (0.10)

Table 2.2. Methylmercury analysis of experimental diets. Values are listed as methylmercury (MMHg), inorganic mercury (Hg II), with both of these combined for total mercury, and the percentage of this total that is methylmercury listed in the last column. Values are expressed in ng/g on a fresh weight basis. Below detection limit is listed as "BD", with one half of this value assigned. There was siginificant differences between treatments for MMHg and THg, but not Hg II.

Treatment	MMHg	Hg II	Total	% MMHg
High Hg diet	186.4256	(BD) 1.26	187.6856	99%
	277.6391	3.6387	281.2778	99%
	242.4151	9.9213	252.3364	96%
Low Hg diet	11.3429	3.7880	15.1309	75%
	4.3210	(BD) 1.125	5.4460	79%
	5.8426	(BD) 1.125	6.9676	84%

Table 2.3. Quality assurance and quality control parameters for metal analysis of experimental diets. Digestion replicate difference is the percent difference between one sample that was digested twice. Diltuion replicate difference is the percent difference between one sample that was diluted twice. Standard refernce material recovery is the percent recovery of the certified published value. Spike recovery is the percent recovery of a spike of a sample with a known value of the element. Method detection limit could not be calucluated because only one digestion or reagent blank was analyzed. Values of the digestion or reagent blank are listed as ppm.

	Al	**Cr**	**Fe**	**Ni**	**Cu**	**Zn**	**As**	**Se**	**Cd**	**Pb**
Digestion replicate differences	5%	6%	6%	5%	3%	4%	5%	4%	3%	17%
Dilution replicate differences	1%	2%	0.7%	7%	0.4%	5%	2%	0.1%	6%	14%
Standard reference material recovery	n/a	100%	101%	90%	97%	104%	121%	144%	103%	94%
Spike recovery	100%	116%	100%	91%	98%	98%	102%	107%	96%	91%
Digestion or reagent blank values (ppm)	3.02	0.01	1.00	0.01	0.001	2.30	0.001	0.001	0.001	0.02

Table 2.4. Metal analysis of experimental diets. Values are means of five replicate anaylses with one standard deviation listed below each value in paratheses. Values are expressed in μg/g on a fresh weight basis. There was siginificant differences between treatments for Se and Ni.

Treatment	Al	Cr	Fe	Ni	Cu	Zn	As	Se	Cd	Pb
High Hg diet	12.8	22.8	64.4	0.091	0.77	7.16	0.10	0.16	0.006	0.03
	(1.05)	(10.8)	(3.2)	(0.01)	(0.05)	(0.58)	(0.01)	(0.01)	(0.001)	(0.01)
Low Hg diet	12.7	22.2	62.3	0.11	0.81	7.33	0.09	0.09	0.005	0.03
	(2.2)	(11.5)	(8.9)	(0.01)	(0.09)	(0.77)	(0.02)	(0.01)	(0.001)	(0.01)

Table 2.5. Tissue mercury concentration and experimental day of death for those crayfish with early mortality. Sex is inidicated by "M" for male and "F" for female. Hg treatments are indicated as "H" for high and "L" for low. Digestive gland is represented by "D. gland", "Gastro" indicates gastrolith and "Exo" indicates exoskeleton. Those values that were not measured or not available to measured are indicated with "n/a". Values are ppm on a dry weight basis.

Exp. day of death	Sex	Age	Hg	Muscle	D. gland	Ovary	Exo.	Gastro	Molt 1	Molt 2	Molt 3	Molt 4
90	F	2	H	6544.92	1915.36	1912.65	1056.38	302.68	20.30	31.16	n/a	n/a
100	F	2	H	7820.54	2933.07	n/a	2990.55	48.34	12.99	54.65	107.22	50.81
114	M	2	H	8435.20	3654.07	n/a	972.74	n/a	24.01	21.81	47.79	n/a
129	F	2	H	6764.27	10130.88	2857.53	2125.99	n/a	0.06	35.46	66.84	n/a
133	F	2	H	6818.74	2081.74	1888.03	805.92	73.68	35.36	70.25	n/a	n/a
136	M	1	H	7777.87	10107.74	n/a	2484.56	n/a	22.08	30.74	45.84	54.20
136	F	1	H	10128.56	4142.62	2974.36	1410.04	65.72	32.92	52.37	151.47	n/a
141	M	2	H	7173.53	4214.39	n/a	1025.92	16.94	6.53	32.41	n/a	n/a
142	M	2	H	8353.16	5292.91	n/a	3012.38	n/a	49.90	54.64	116.14	80.62
73	M	2	L	319.76	400.50	n/a	241.15	0.06	9.79	27.15	n/a	n/a
137	M	2	L	286.77	786.99	n/a	212.95	n/a	53.21	34.36	23.42	n/a

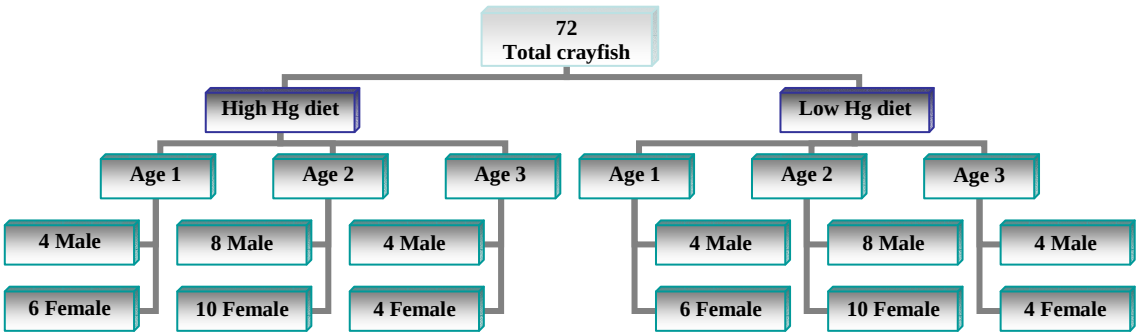

Figure 2.1. Organizational chart of experimental design.

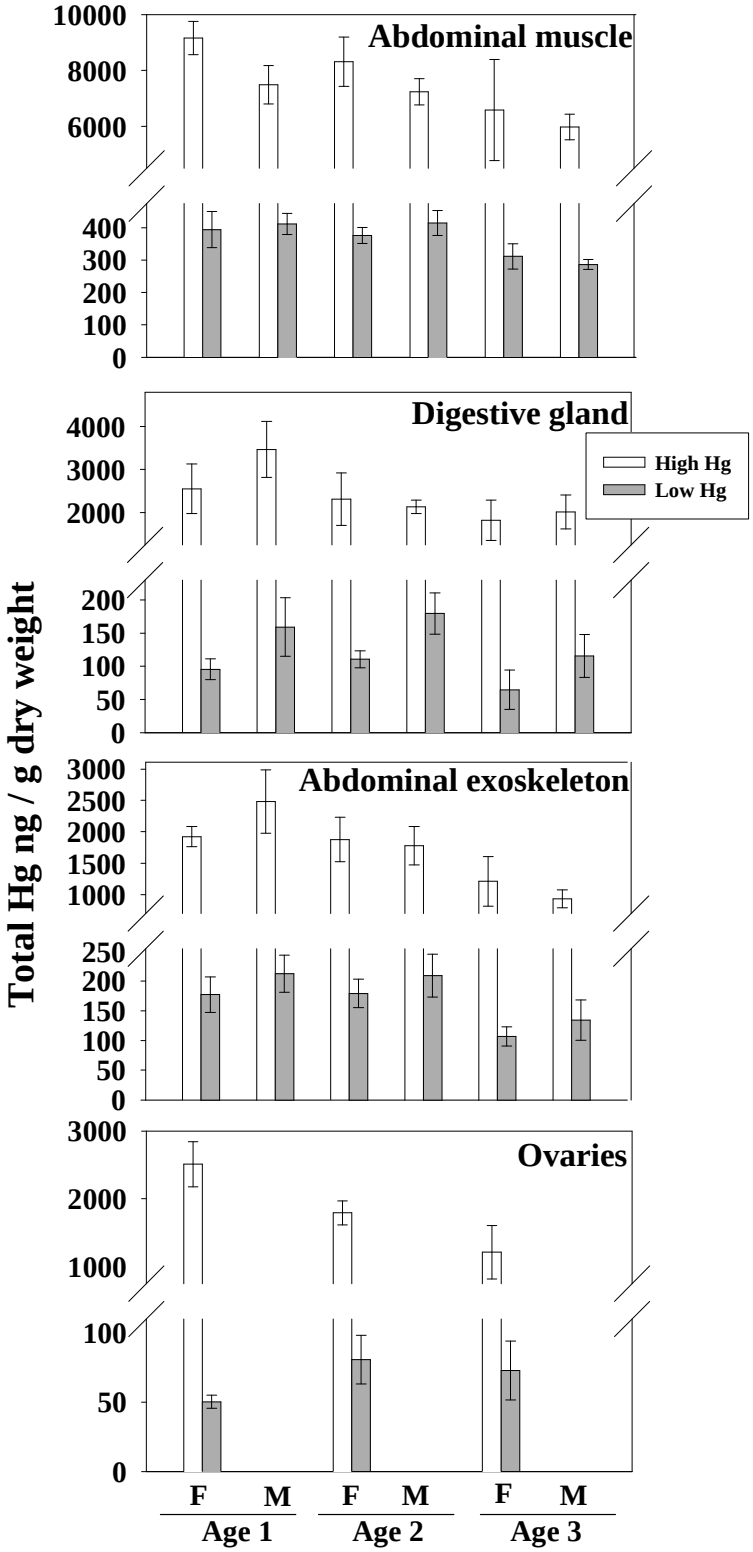

Figure 2.2. Mean Hg accumulation in the abdominal muscle, digestive gland, abdominal exoskeleton and ovaries by Hg exposure, sex and age. Bars represent mean ± one standard error.

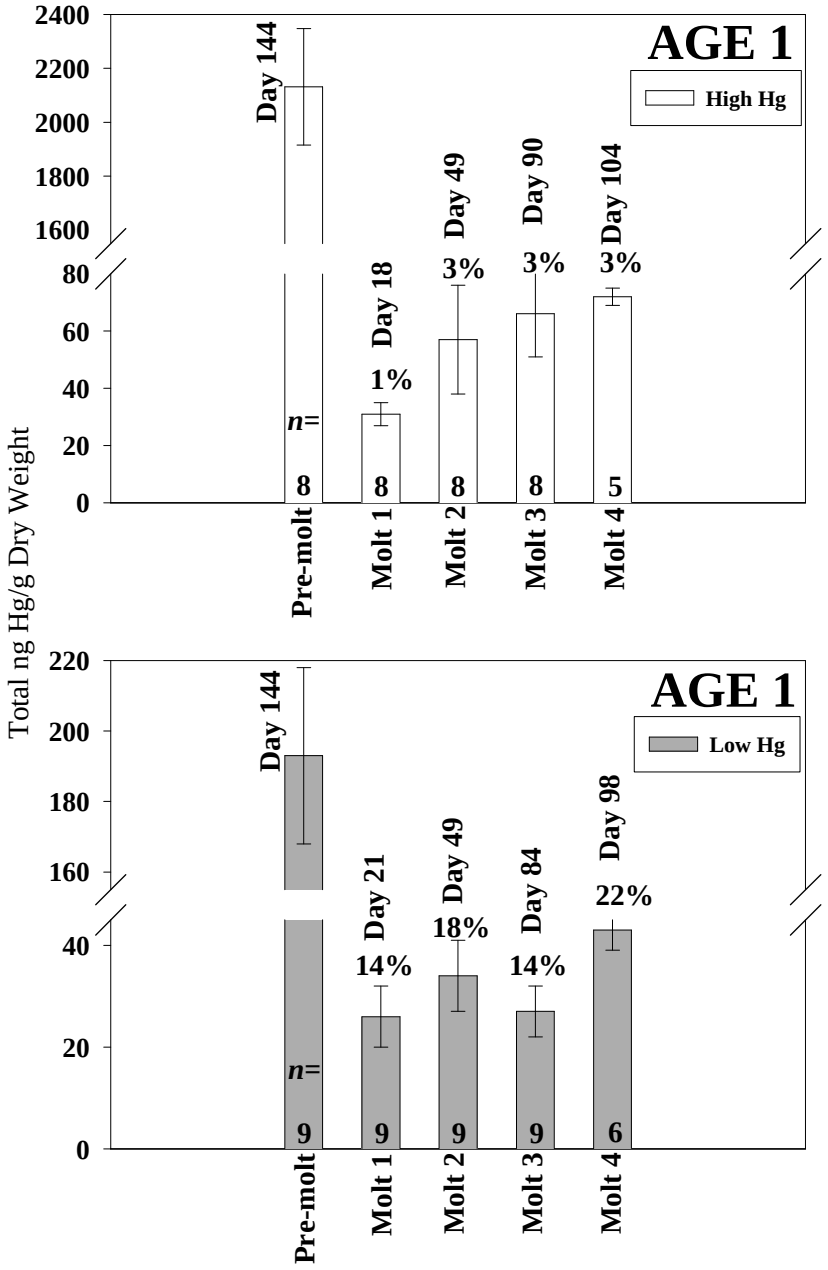

Figure 2.3. Mean exoskeleton and molted exoskeleton Hg accumulation by exposure for age one (youngest). Hg in pre-molt exoskeleton greatly exceeded Hg in the molted exoskeleton for both high and low Hg exposures. These data were calculated by averaging Hg concentrations for both sexes in each group (high and low separately) as pre-molted exoskeleton and post molt exoskeleton. Pre-molted exoskeleton is defined as the abdominal exoskeleton that was taken from each crayfish when euthanized (day 142). Post-molt exoskeleton (molt 1-4) is defined as the abdominal exoskeleton that was shed away from the body during earlier parts of the experiment. Bars represent mean $\pm$ one standard error. Within each bar the number of individuals that were included is listed. The mean day for each molt is listed above the representative bar

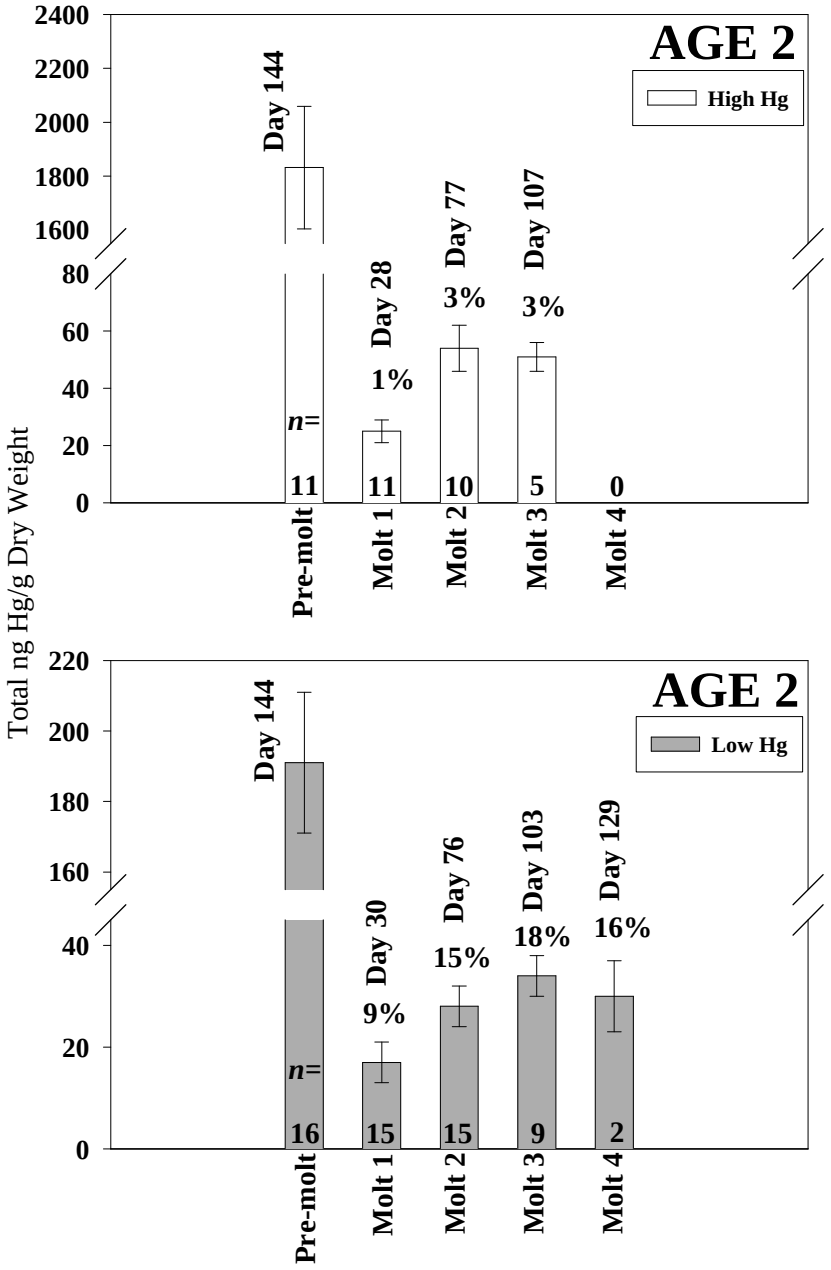

Figure 2.4. Mean exoskeleton and molted exoskeleton Hg accumulation by exposure for age two (middle). Hg in pre-molt exoskeleton greatly exceeded Hg in the molted exoskeleton for both high and low Hg exposures. These data were calculated by averaging Hg concentrations for both sexes in each group (high and low separately) as pre-molted exoskeleton and post molt exoskeleton. Pre-molted exoskeleton is defined as the abdominal exoskeleton that was taken from each crayfish when euthanized (day 142). Post-molt exoskeleton (molt 1-4) is defined as the abdominal exoskeleton that was shed away from the body during earlier parts of the experiment. Bars represent mean $\pm$ one standard error. Within each bar the number of individuals that were included is listed. The mean day for each molt is listed above the representative bar

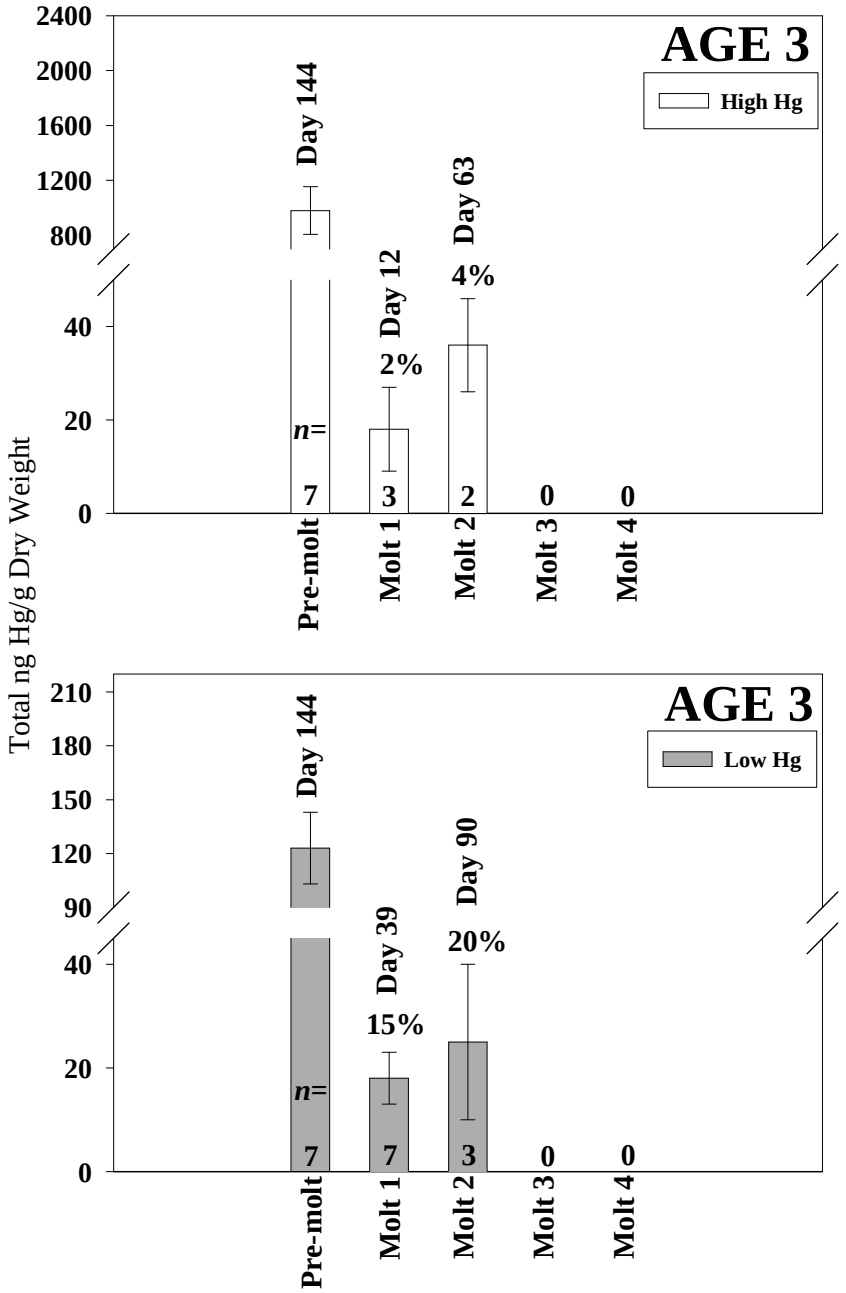

Figure 2.5. Mean exoskeleton and molted exoskeleton Hg accumulation by exposure for age three (oldest). Hg in pre-molt exoskeleton greatly exceeded Hg in the molted exoskeleton for both high and low Hg exposures. These data were calculated by averaging Hg concentrations for both sexes in each group (high and low separately) as pre-molted exoskeleton and post molt exoskeleton. Pre-molted exoskeleton is defined as the abdominal exoskeleton that was taken from each crayfish when euthanized (day 142). Post-molt exoskeleton (molt 1-4) is defined as the abdominal exoskeleton that was shed away from the body during earlier parts of the experiment. Bars represent mean $\pm$ one standard error. Within each bar the number of individuals that were included is listed. The mean day for each molt is listed above the representative bar

CHAPTER 3

CHRONIC DIETARY METHYLMERCURY EXPOSURE ON THREE JUVENILE STAGES OF THE CRAYFISH *PROCAMBARUS CLARKII*: BEHAVIORAL AND PHYSIOLOGICAL CHANGES

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CHAPTER 3

CHRONIC DIETARY METHYLMERCURY EXPOSURE ON THREE JUVENILE STAGES OF THE CRAYFISH *PROCAMBARUS CLARKII*: BEHAVIORAL AND PHYSIOLOGICAL CHANGES

ABSTRACT

Wetlands are critical in providing foraging and breeding habitat for a multitude of organisms. Atmospheric depositions of mercury (Hg) along with conditions favoring Hg methylation in these wetlands are major causes of elevated Hg concentrations in biota. Red swamp crayfish (*Procambarus clarkii*) are abundant in wetlands in the southeastern US, and are ideal model organisms to study Hg accumulation and potential sub-lethal effects. The effects of chronic low-level exposure via the diet are poorly researched; therefore, we exposed juvenile crayfish (n=72) of known age and sex to diets containing relatively high and low concentrations of methylmercury (MMHg) using a 2x2x3 randomized block design. The low Hg diet averaged 9 ppb Hg fresh weight (80% MMHg) while the high Hg diet averaged 278 ppb Hg fresh weight (98% MMHg). Sub-lethal effects after 142 days of chronic dietary exposure included alterations in behavior and growth, with severity varying based on sex and age. Behavioral alterations in the crayfish fed the high Hg diet included an almost doubled time to find shelter/burrow when compared to the crayfish fed the low Hg diet. Growth suppression occurred within the youngest and oldest males and the oldest females fed the high Hg diet. In nature, these effects could reduce crayfish survival.

INTRODUCTION

Although atmospheric inputs of mercury (Hg) into wetlands are relatively small, organisms inhabiting wetlands can accumulate relatively high concentrations of Hg because elimination is very slow relative to uptake [1, 2]. Biota living in wetlands may thus be exposed to relatively low Hg concentrations in food, water and sediment during their entire lifespan. The potential consequences of chronic dietary exposure are poorly understood in benthic invertebrates, which form the base of many wetland food webs. The red swamp crayfish *Procambarus clarkii* was chosen as a model organism to study effects of chronic Hg exposure because of its relatively wide distribution, importance as a food item for both humans and wildlife, and potential for exposure to Hg in wetlands in the southeastern U.S.

While the mercury that is deposited from the atmosphere is largely inorganic [3, 4], the form that accumulates in biota is largely methylmercury [5, 6]. Thus, methylation is a key process affecting mercury cycling, bioavailability and accumulation. Many wetlands that are inhabited by *Procambarus*, whether natural or commercially cultivated, tend to have factors that favor high methylation rates, making them especially sensitive to mercury inputs [7-10]. Recent studies have indicated that periodic flooding and drying of a wetland can enhance methylation, and therefore increase mercury concentrations in resident organisms [9, 10]. This suggests that repeated flooding and drying of the commercially cultivated crayfish farm ponds has the potential to increase mercury bioaccumulation and possibly cause adverse effects to the crayfish and their predators, including humans.

The diet is likely the most important route of Hg exposure to crayfish in their natural environment, and laboratory exposures have shown efficient accumulation with very slow

depuration rates of both total Hg and MMHg [11, 12]. The diet of crayfish in the natural environment is highly variable and influenced by multiple factors [13]. Crayfish are opportunistic omnivores, feeding on algae, detritus, and animal matter. The contribution of each of these components to the diet varies with availability, and also varies with the age and sex of the crayfish [13]. Juvenile crayfish prefer a higher proportion of animal protein, whereas adult diets contain higher proportions of algae and moss [13]. Sex differences play a role when food with high nutritional value becomes scarce, because males are typically much larger than females and therefore dominate nutritional resources during food scarcity [13]. Variability of the crayfish diet could contribute significantly to variation in Hg exposure, along with variation in the proportion of MMHg in the diet.

Given their habitat and dietary habits, crayfish in the natural environment have the potential to be exposed to relatively low concentrations of Hg in their diet over their entire lifespan, with a majority (typically > 50%) of the Hg as Hg II [14-16]. However, juvenile crayfish prefer animal protein, such as fish [13], which would expose them to higher concentrations and proportions of MMHg than a detritus diet. The high neurotoxicity of methylmercury may cause adverse behavioral or physiological effects at concentrations far below exposure levels that cause mortality, especially during early development [2]. Sub-lethal chronic dietary exposures and the potential health and behavioral effects due to Hg exposure are poorly researched in benthic crustaceans. Mercury in the diets of crayfish of different sexes and ages may alter normal health, behavior and growth. Therefore, we examined the sub-lethal effects of chronic dietary Hg exposure to *P. clarkii*.

MATERIALS AND METHODS

Preparation of experimental diets, crayfish husbandry and feeding, and analysis of mercury in crayfish and the diets are described in chapter 2. Crayfish of known age (by developmental stage) and sex were obtained by breeding adults obtained from a local crayfish farmer. The juveniles were categorized into three distinct age classes, all within 3-4 weeks of each other. Age one, the youngest, were approximately three weeks old at the beginning of the feeding experiment and had reached the fourth molt. Age two, the middle age group, were approximately five weeks old at the beginning of the experiment and had reached the sixth molt. Age three, the oldest age group, were approximately eight weeks old when the feeding experiment began and had reached the eighth molt.

The experiment used a 2X2X3 randomized block design (2 diet treatments, 2 sexes, 3 age classes) with a total of 72 crayfish. Briefly, crayfish were fed artificial diets *ad lib*, and checked for molting daily. Diets contained an average of either 9 ppb Hg fresh weight (80% MMHg) or 278 ppb Hg fresh weight (98% MMHg). A cube of diet was placed in each container with an individual crayfish, and renewed when it had been nearly consumed. Each cube was weighed before feeding. Growth was monitored by weighing the crayfish every seven days. Weight data was then used to compare growth between the two Hg exposures, among sexes and age classes, and to calculate hepatopancreatic index. Mass of the crayfish and food consumption also allowed for the calculation of the dietary mass conversion efficiency (MCE) as weight of food fed (g) / weight of animal (g) X 100. Crayfish were fed the high or low mercury experimental diets for 142 days; and behavioral experiments were conducted at 139 days. Crayfish were euthanized and dissected at 142 days. Tissues (abdominal muscle, abdominal exoskeleton,

ovaries if present, gastroliths if present, and the digestive gland) were collected and analyzed for total Hg (THg).

Behavior Trials

To evaluate the effects of dietary mercury exposure on crayfish behavior, we chose a simple, quantifiable response. Juvenile crayfish tend to avoid bright light, and when placed in an open, lighted location, they move away from the light to seek refuge in dark shelters [17-19]. Behavior was quantified as the amount of time each individual took to find and enter shelter once placed in a lighted environment. Behavior was also assessed by videotape analysis of forced escape responses from the shelter area. The behavior trials were modeled after Cromarty *et al*. [20], and were conducted in a 209L experimental tank that was designed with a light to dark gradient (Figure 3.1). The tank was maintained with room temperature (16 degrees C) well water, and contained twelve tubes (made of PVC pipe) for hiding located in the darkest part of the tank. Crayfish were randomly selected and placed in a fixed location 10 cm above the water at the bright end of the tank. The trials began by releasing the crayfish into the water, and the total time for each crayfish to seek refuge within a tube was measured with a stopwatch. The trial was terminated after the crayfish had entered a tube, or if six minutes passed without the crayfish finding shelter. After the crayfish entered the shelter tube, they were prodded with a metal probe through one of the three holes located at the top of each tube to elicit an escape response (Figure 3.2). The crayfish escape response is also a highly stereotyped behavior [21, 22], involving one or more tail flips that propel the crayfish rapidly away from the stimulus. The escape response to prodding was videotaped for later analysis.

Videotape Analysis

Cameras were placed in horizontal and vertical positions so that the experiments were simultaneously recorded from two perspectives on two video recording systems. Video recording of each crayfish behavior trial was then analyzed frame-by-frame. Each lasted 0.03 seconds. The following characteristics of the escape response were measured for each crayfish in subsequent viewing of the recordings: distance traveled (m), number of tailflips (Tf), duration of the response (s), and frequency of tailflips (Tf/s). These measurements were used to calculate velocity (m/s), acceleration (m/s/s), force (N; (kg * m/s/s)], work (J), distance traveled/weight/tailflip (m/kg/Tf), and distance traveled/weight (m/kg), based on the methods of Cromarty *et al.*, [20]. The latter parameters were a crude calculation to determine whether individual crayfish variability in weight and size altered the parameter. Because of lengthy and erratic escape responses of some of the crayfish, total distance traveled was determined for all the crayfish by placing a clear sheet of plastic over the viewing screen and tracing the crayfish movements with a marker on the plastic sheet. The sheet was then removed and a string was placed over the tracing and measured for total length. The tip of the tail was used as a point of reference for each measurement. The number of tailflips was counted according to Figure 3.2. A complete tailflip is defined as beginning immediately after the start of abdominal flexion and ending at abdominal extension (Figure 3.2; sequence a-f) [20]. The duration of the entire response was recorded by time posted directly on the videotape.

Statistical Analysis

All statistics were performed using SAS for Windows (version 8.1; Cary, NC, USA). Data were checked for normality before analysis using Sharpiro-Wilk's tests. Bartlett's test was used to verify homogeneity of variance. A three-way ANOVA with block effects was used to

determine differences between treatments for growth data. An ANCOVA with weight as a covariate was used to determine differences between the two Hg exposures for the time to find shelter and the escape response parameters. The oldest (age 3) females were corrected prior to statistical analysis by removing outliers according to the methods of Grubbs *et al.*, [21]. The high Hg exposure originally had one female with an unusually high weight, and the low Hg exposure had one female with an unusually low weight.

RESULTS

There was a significant difference in mass conversion efficiency (MCE) among ages ($p=0.0008$, $F_{11, 47}=8.31$) and Hg treatments ($p=0.0033$, $F_{11, 47}=9.60$), but not sexes (Figure 3.3). Figure 3 illustrates the MCE by Hg exposure, sex and age. The bars represent mean $\pm$ one standard error. The younger age class tended to have a higher MCE than the older age class. There was a marginally significant interaction between sex and age ($p=0.0590$, $F_{11, 47}=3.01$) as shown in Figure 3.3. The significant interaction is due to the responses of the age class one males and the age class three males and females.

The hepatopancreatic index (HI) is a measure of the hepatopancreas weight (fresh weight basis), divided by the fresh weight of the organism, and then multiplied by one hundred (Figure 3.4). The HI's were not significantly different among diet treatments or ages; but did differ between sexes ($p=0.0004$, $F_{4, 52}=14.60$). The females had higher average HI's than males. Figure 3.4 illustrates the HI by age, sex and Hg exposure. Each bar represents the mean $\pm$ one standard error.

Figures 3.5 and 3.6 represent the growth by weight measured every seven days. The males fed the high Hg diet had reduced growth in two of the 3 age groups (Figure 3.5); however, crayfish weight at the end of the experiment did not differ between diet treatments in any age

group. Calculated slopes (representing growth as change in weight over time) for the high and low Hg exposures ranged from 0.01-0.03 and 0.03-0.05, respectively. Slopes were significantly different between the two Hg diets for age 1 ($p<0.0001$, $F_{3,\ 143}=22.44$) and age 3 male crayfish ($p<0.0001$, $F_{3,\ 164}=8.16$). The oldest group (age 3) gained an average of two grams during the experiment when fed the high Hg diet, but an average of six grams when fed the low Hg diet. The youngest (age 1) group gained an average of two grams when fed the high Hg diet, and four grams when fed the low Hg diet.

The growth of females differed between diets only in the oldest age group (age 3; Figure 3.6). This age group was corrected prior to statistical analysis by removing outliers according to the methods of Grubbs *et al.*, [23]. The high Hg exposure originally had one female with an unusually high weight, and the low Hg exposure had one female with an unusually low weight. End weights differed between dietary treatment in the age 3 females ($p=0.0192$, $F_{3,\ 4}=14.42$), as did slopes ($p=0.0016$, $F_{3,\ 122}=10.48$). The age 3 females gained an average of 0.5 grams during the experiment when fed the high Hg diet, but an average of 2.5 grams when fed the low Hg diet. Calculated slopes for the two Hg exposures ranged from 0.01-0.04 and 0.02-0.03. There was no difference in weight at the end of the experiment or growth rates as indicated by slope between dietary Hg treatments for the other two age groups.

Growth, as determined by comparing slopes, was significantly different between sexes only in the youngest when fed the high Hg diet ($p=0.0005$, $F_{3,\ 164}=12.71$). All other differences between sexes were not significant, regardless of age or diet treatment.

Because time to shelter might vary with crayfish size, weight was used as a covariate. Time to shelter differed between Hg treatments ($p=0.0071$, $F_{2,\ 60}=7.77$), but not among sex or age groups (Figures 3.7 & 3.8). ANCOVA indicated that weight and treatment-weight

interaction were not significant. Crayfish fed the low Hg diet took approximately half the time to find refuge as those fed the high Hg diet (Figure 3.7). Each bar within Figure 3.7 indicates the mean (± one standard error) amount of time (in seconds) that the crayfish from each Hg exposure took in order to find shelter. Differences between sex and age were not detected, so these groups were pooled to test for treatment effects. While differences among ages and sexes were not significant, lots by age, sex and Hg exposure show trends for some groups (Figure 3.8). Among females fed the high Hg diet (Figure 3.8), those from the first and third age groups took longer to find refuge as compared to the females of the same age fed the low Hg diet. Among males fed the high Hg diet, those from the first and second age groups took considerably longer time to find shelter when compared to the males of the same ages but fed the low Hg diet.

There was no difference between the two diet treatments for distance, duration, velocity or acceleration in the tailflip escape response (Figure 3.9). Figure 3.10 shows that there was no statistically significant difference between the two Hg treatments for force or work. There was also no difference between the two Hg treatments for the number of tailflips, frequency of tailflips, distance corrected for weight, or distance by weight by tail flip (Figure 3.11).

DISCUSSION

Mass conversion efficiency (MCE) is an index of how efficiently food is converted into somatic tissue. This is a concern for commercial cultivators, and may have ecological implications for wild populations if MCE is affected by stressors. In the present study, significant interactions between sex and age and response to dietary mercury were noted. The younger age class tended to have a higher MCE than the older age class. This is related to the demands of isometric growth. Juvenile crayfish undergo rapid growth and molting until sexual maturity is attained. The youngest age group (age 1) therefore displays the highest MCE, with

the oldest age group (age 3) approaching sexual maturity, displaying the lowest MCE. Figure 3.3 shows that MCE was significantly lower for crayfish in the first and third age groups fed the high Hg diet. This suppression in the youngest age group (age 1), is largely driven by the drastic difference between the males in the two Hg exposures (Figure 3.3). Females in the age 1 group did not respond to the high Hg diet in the same manner. However, in the oldest age group (age 3) MCE is lower for both sexes fed the high Hg diet. As mentioned earlier, crayfish growth is typically isometric, but can also be allometric, where parts of to body increase disproportionately to the rest. Rhodes and Holdich [24] found that the external sexual dimorphism of secondary characters is usually isometric in juveniles and adults until approaching sexual maturity. In crayfish approaching sexual maturity, male chelae and female abdomens grow allometrically faster relative to other body parts. The male chelae are a symbol of hierarchy status, and also serve as a means to dominate in altercations with other males in order to mate with receptive females. The female disproportional abdominal increase is also a function of reproduction in that an increase in abdominal size is necessary for carrying eggs.

When fed a diet low in mercury, MCE's at ages 1 and 2 are similar between males and females. However, at the oldest (age 3), there is a significant difference in between the sexes even in the low Hg diet treatment; the females have a lower MCE. Even though variations occur between sexes in growth of certain body parts, female *P. clarkii* are still about half the size of males when sexual maturity is reached [25].

The lower MCE in the youngest males fed the high Hg diet (age 1) (Figure 3.3), could be a function of isometric growth demands and/or impaired function of the hepatopancreas. The hepatopancreatic index (HI), is a measure of how well the organ is in proportion to its body size. Alterations of this proportion could be indicative of impaired function of the organ. The

difference between the HI for the sexes has been previously documented for another freshwater crayfish, the marron (*Cherax tenuimanus*) [26]. This previous work suggested that the hepatopancreas is large in female freshwater due to the energy requirements of oogenisis [26]. Although the only statistically significant difference found in the present study was between sexes, the youngest males had a somewhat reduced HI when fed the high Hg diet. Pervious work suggests that Hg can alter the ability of the hepatopancreas in crayfish to replenish energetic reserves (examples: total lipids and caloric content) after starvation [27]. Jewell *et al*., [28] also observed that the crayfish hepatopancreas generates oxygen radicals during microsomal electron transport, a process that is known to be altered by Hg [29]. Either alteration in oxidative metabolism or lipid reserves might influence HI in the youngest males fed the high Hg diet. The lower MCE in the youngest males fed the high Hg diet, coupled with the lower hepatic index in this group, may lead to the reduced growth rate as seen in Figure 3.5.

Males fed the high Hg diet had stunted growth in the youngest and the oldest age groups (Figure 3.5), while only females the oldest age group showed decreased growth when fed the high Hg diet. Another factor that could contribute to decreased growth is inhibition of cell division. MMHg disrupts the spindle apparatus and has been shown to cause chromosome breakage in plants [30]. The suppression of growth with high dietary Hg for both males and females in the oldest and youngest age groups fits well with the observation that MCE is also lower in these groups. This also lends evidence to the idea that the both age groups are undergoing a crucial time for growth. As explained earlier, the youngest age males could be undergoing exponential growth. The oldest age group is nearing sexual maturity. Their growth is likely allometric, since the female abdomen and the male chelae are disproportionably growing in relation to the rest of the body.

Although growth was affected in the males and females, there was no statistically significant difference in the number of days between molts for either sex, regardless of Hg concentration in the diet [Chapter 2]. Likewise, there was a no statistically significant difference for the number of molts per individual between dietary treatments for either sex. The number of molts differed among ages, but not between dietary Hg treatments or sexes. This relationship does not follow the differences in growth patterns we observed with dietary Hg exposure. The relationship follows the general pattern of the youngest growing at a faster pace than the older, and therefore molting more frequently. However, the molting did not reflect the decreased growth observed in males and females fed the high Hg diet, regardless of age.

The lack of differences in molting frequencies between sexes suggests that there should be little difference between these groups in Hg excreted to the exoskeleton and shed during molting. The only significant factor influencing to the amount of mercury deposited in to the shed exoskeleton was the dietary mercury treatment; age and sex differences were not significant. There were significant differences in mercury concentrations in shed exoskeleton between the Hg treatments for molt series two thru four.

Time to shelter behavior differed between Hg treatments, but not among sexes or age groups (Figure 3.7). Crayfish fed the low Hg diet took approximately half the time to find refuge as compared to those fed the high Hg diet. This suggests impairment of sensory and/or locomotion function, and potential for reduced survival in the natural environment. If mercury impairs the ability to seek and find shelter, the individual is more likely to be eaten, and more likely to pass on the body burden of mercury it possesses to predators.

To further examine any potential impairment of neurologically-mediated functions of crayfish, videotape analysis of their escape response was conducted. The escape response is

mediated by 5-HT [22], a neurotransmitter that is also involved in ovarian maturation [22, 31]. While evidence exists for potential disruption of the neurotransmitter by Hg [31], there was no difference between the two diet treatments for any parameter of the tailflip escape response.

Sub-lethal effects on juvenile crayfish exposed to ecologically relevant dietary concentrations [32] include growth and behavior alterations, with severity dependent on age and sex. These alterations definitely affect not only the individual and possibly the population, but could indirectly effect predators. The crayfish that were exposed to higher Hg concentrations took longer to find shelter, and therefore have a higher potential of passing the body burden on to a potential predator.

CONCLUSIONS

Based on measurement of multiple endpoints, younger male crayfish seem to be most sensitive to Hg exposure. Changes in growth may be a concern for the young males, but also the sexually-maturing (age 3) juvenile males and females. The reduced growth during a time when rapid growth is especially crucial has negative implications not only for an individual, but to the population. Although mercury is a suspect endocrine disrupter, we observed no difference in molting frequencies, a hormonally mediated process. Results suggest that behavior may be a sensitive indicator of mercury toxicity in crayfish. Alterations in each endpoint studied may have negative impacts to the individuals. The summation of these impacts suggests that environmentally-realistic dietary Hg exposure may have deleterious effects on crayfish populations.

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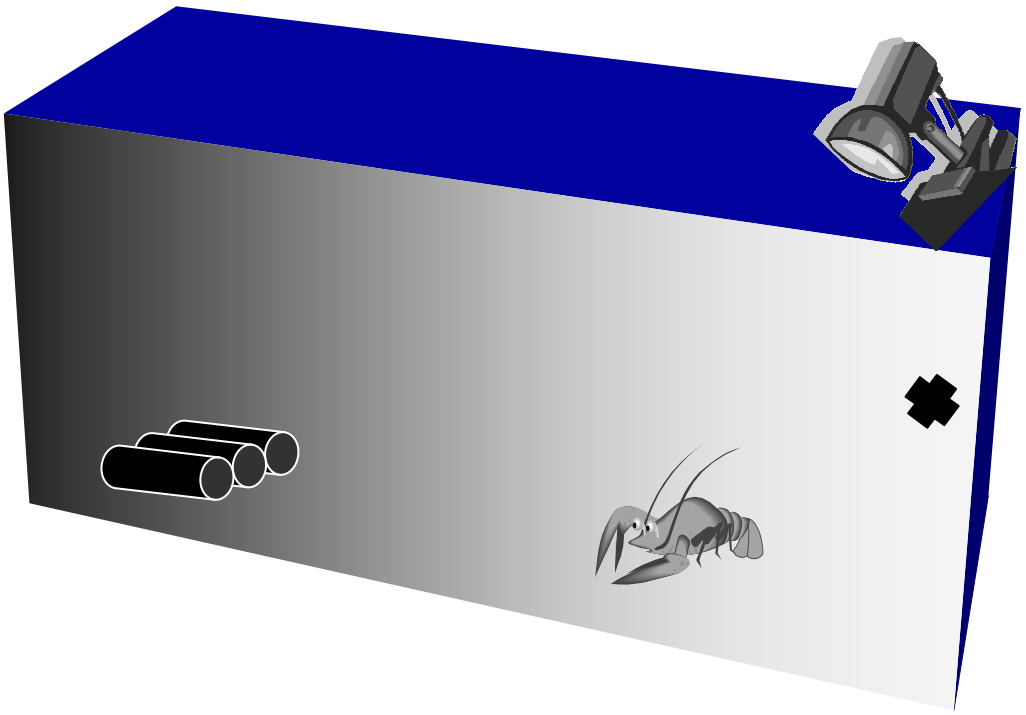

Figure 3.1. Diagram of behavior trials. Trials began by placing crayfish at the same point of origin (indicated by black X on diagram) within a 209 L liter tank. The tank contained a light to dark gradient, with 12 tubes representing burrows/shelter in the darkest part of the tank. The time the crayfish took to find shelter was recorded and later compared between the ages, sexes and exposures.

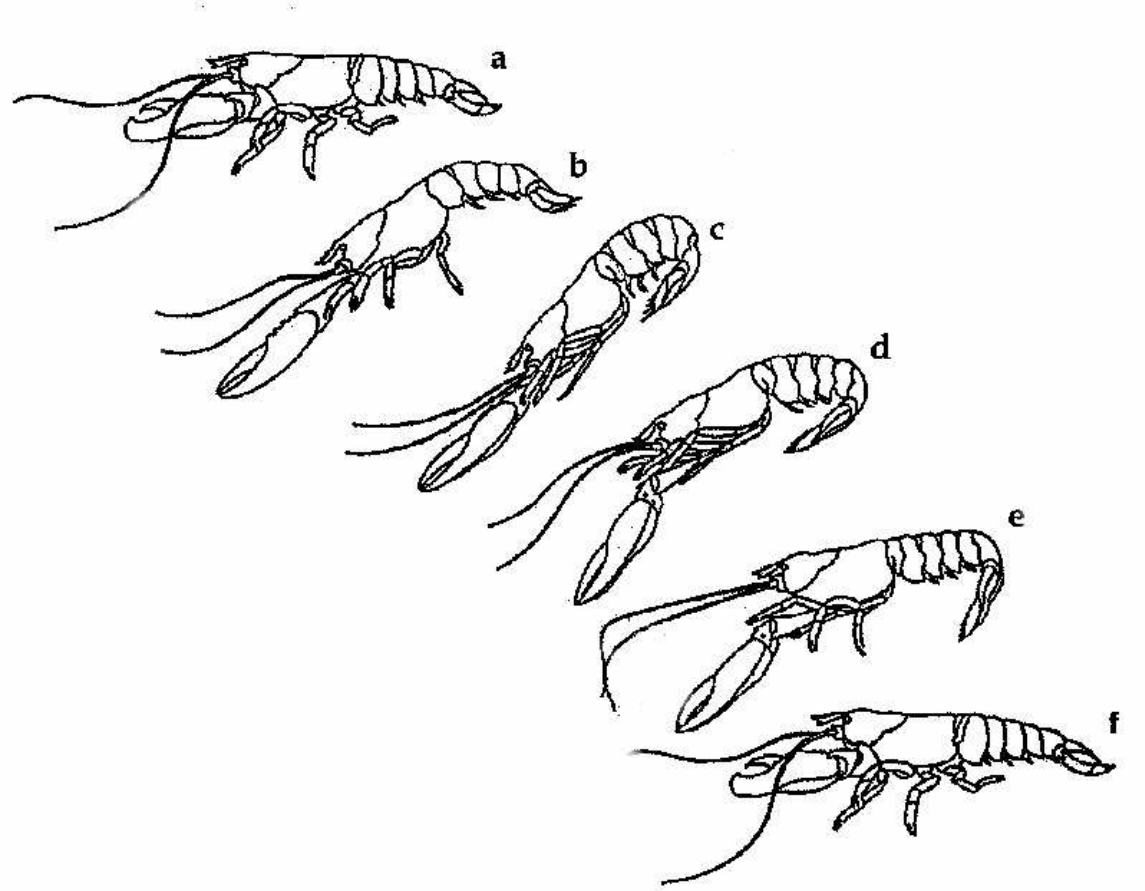

Figure 3.2. Diagram of escape response. Diagram credited to K. Davignon, Graphics specialist, URI, from the works of Cromarty, *et al*., [20].
(a) = beginning of swim; (f) = end of a single tailflip

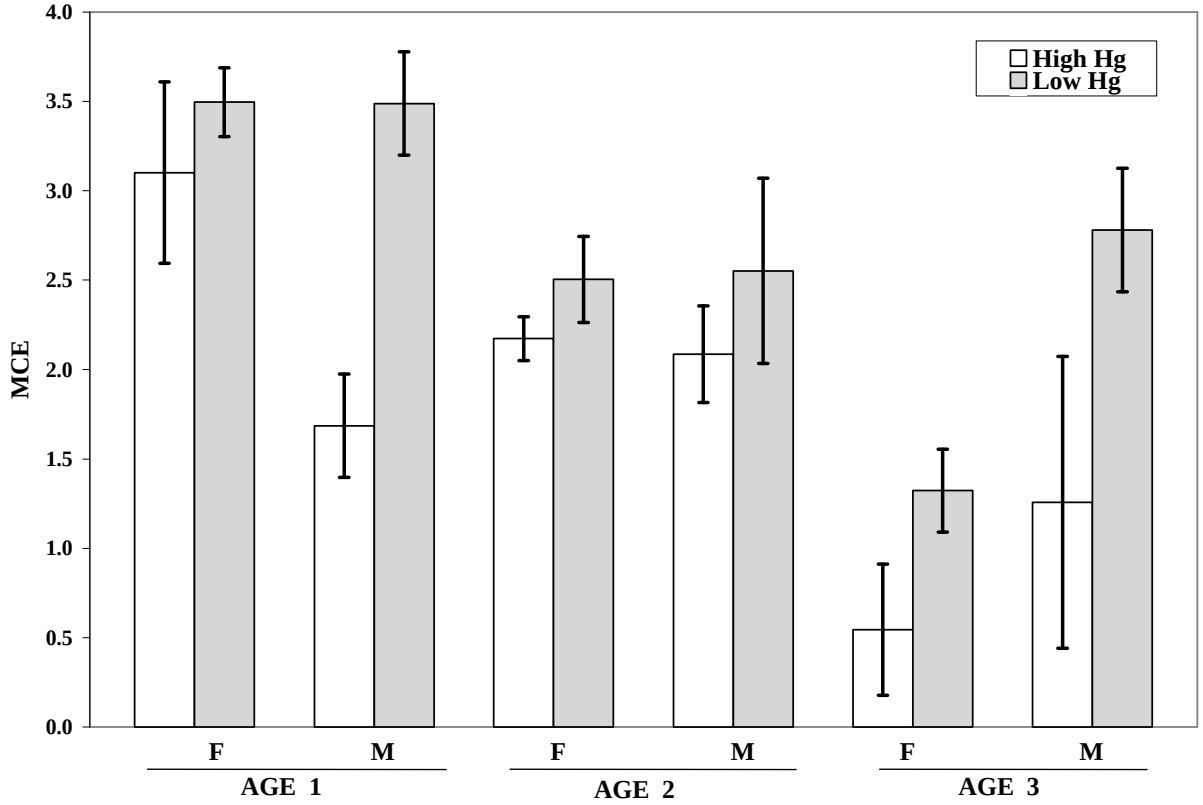

Figure 3.3. MCE (mass conversion efficiency) by Hg exposure age and sex. The mass conversion efficiency (MCE), is defined as the amount of weight gained divided by total food eaten multiplied by 100. Bars represent mean $\pm$ one standard error.

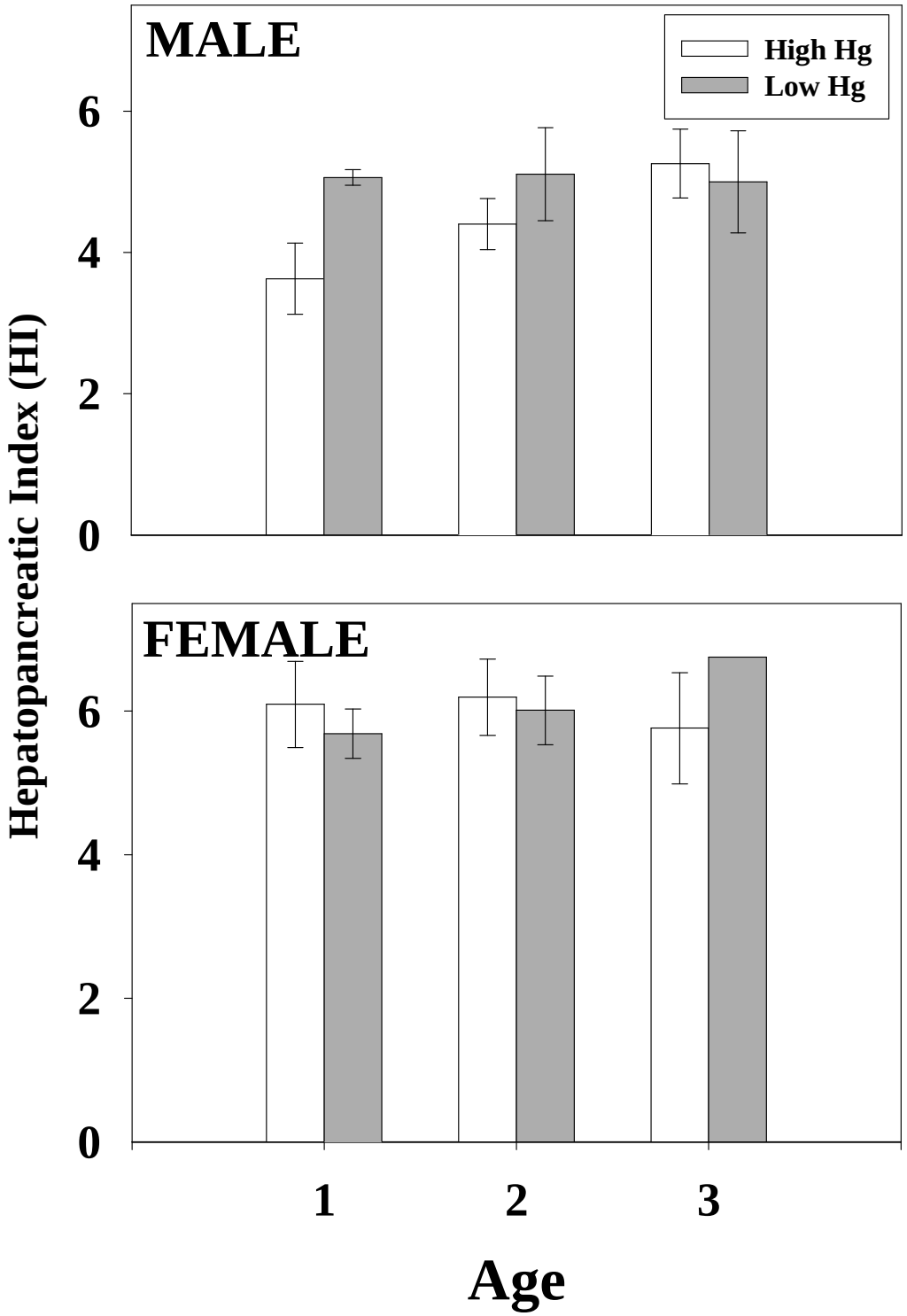

Figure 3.4. Hepatopancreatic index for males and females of both Hg exposures by age. The hepatic index (HI) is a measure of the hepatopancreas weight (fresh weight basis), divided by the fresh weight of the organism, and multiplied by 100. Bars represent mean $\pm$ one standard error.

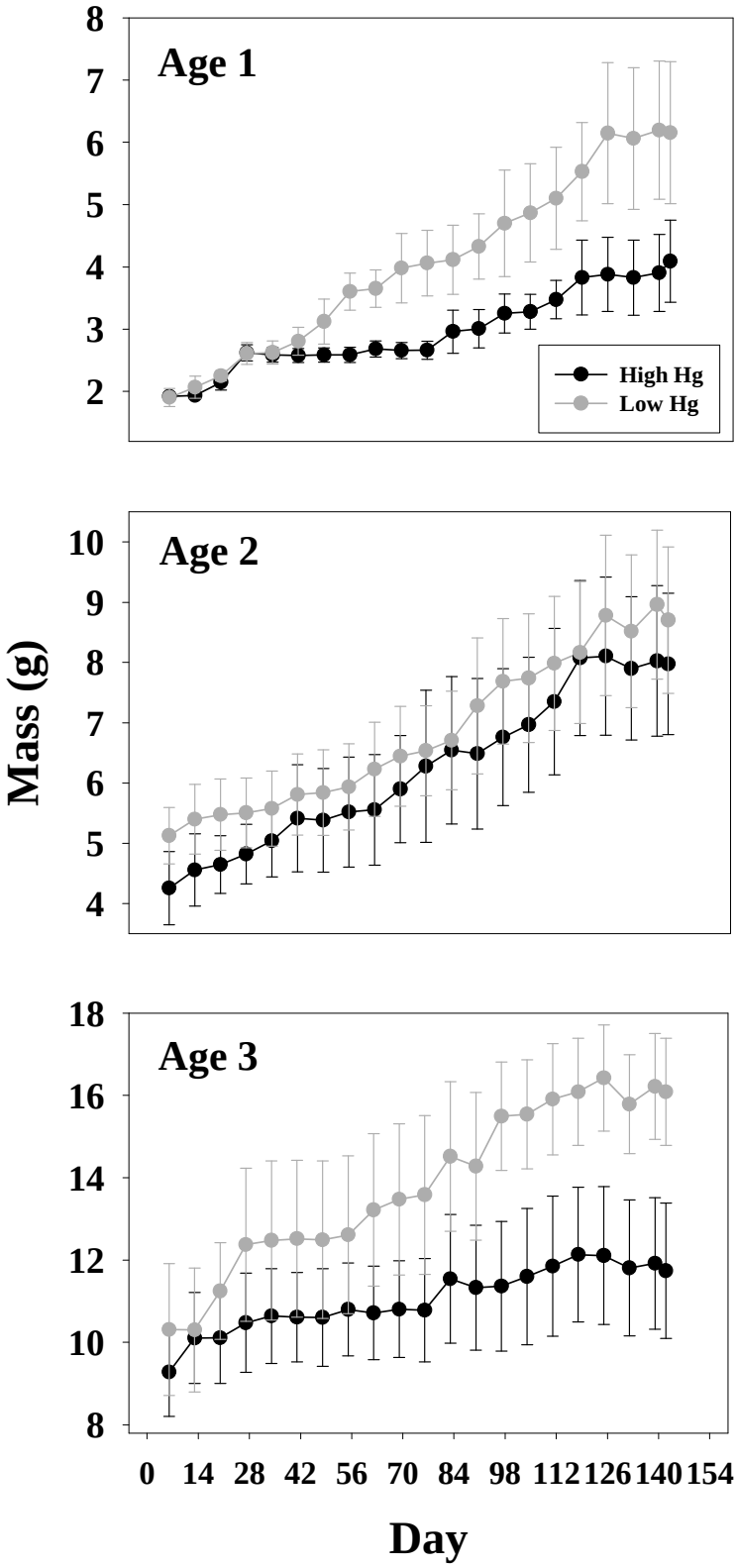

Figure 3.5. Average growth by Hg exposure and age for **MALES**. Bars represent mean ± one standard error.

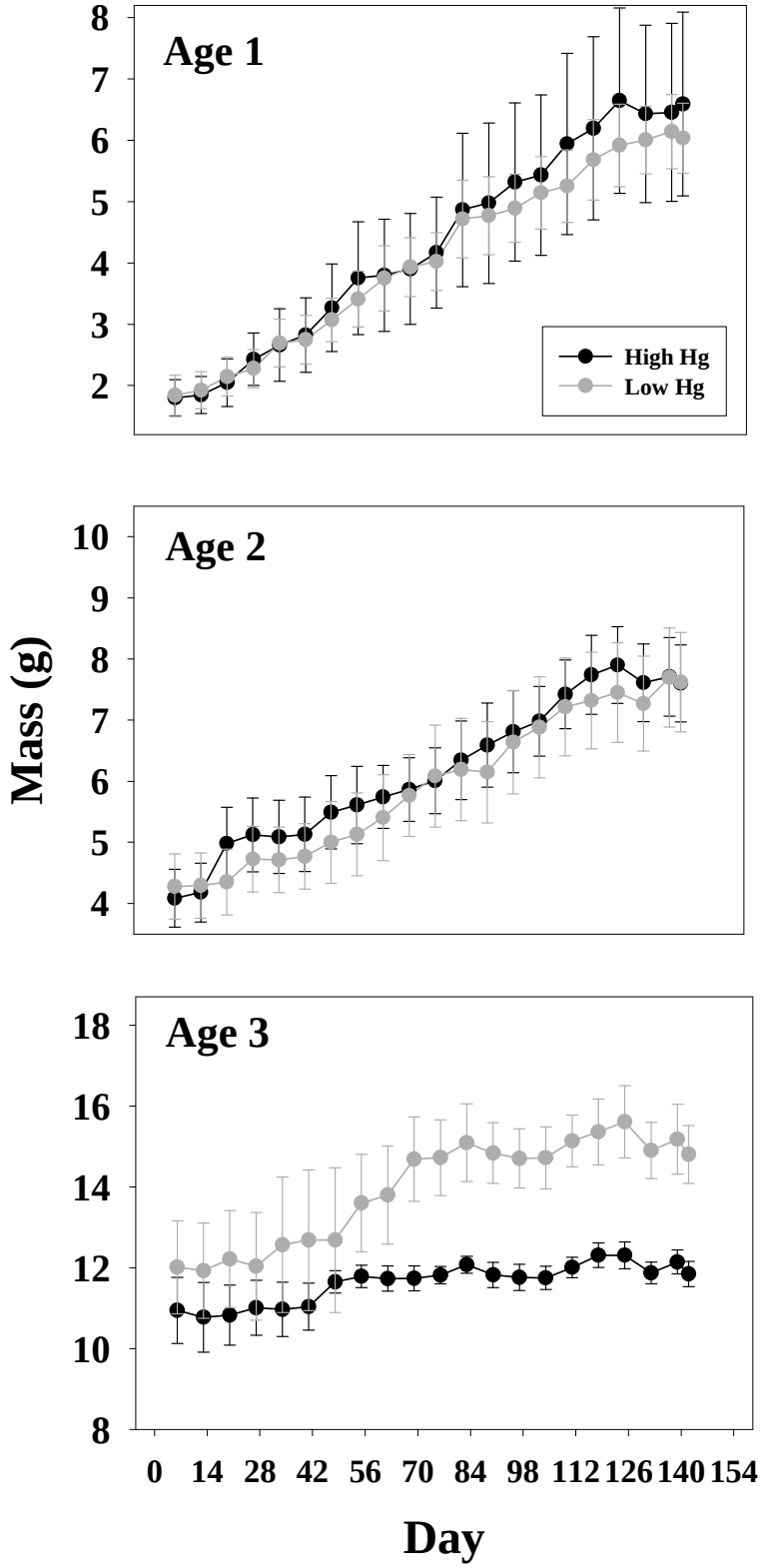

Figure 3.6. Average growth by Hg exposure and age for **FEMALES**. Bars represent mean ± one standard error. Age 3 insert removes one outlier from each exposure as judged by Grubbs [21].

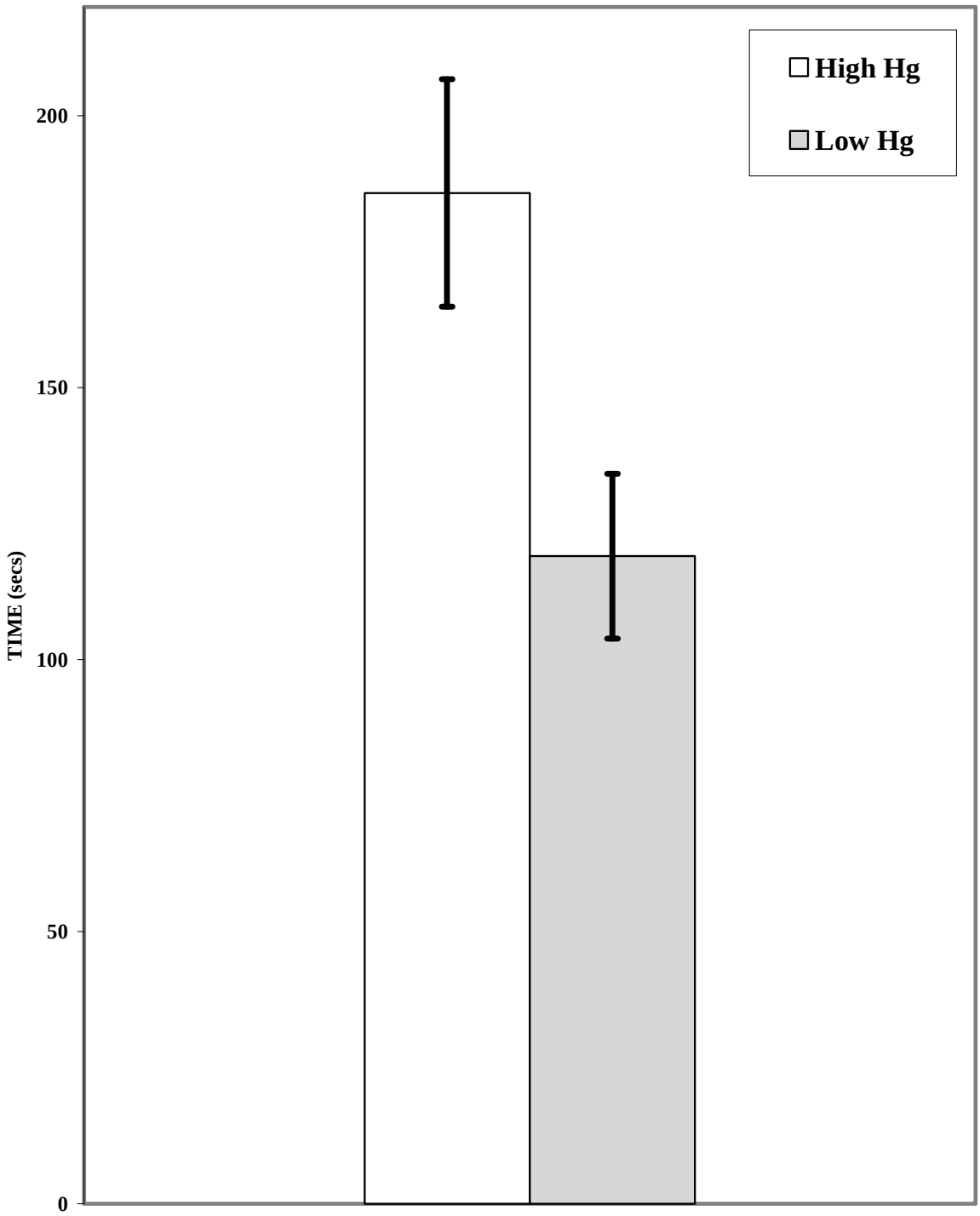

Figure 3.7. Average time to seek shelter by Hg exposure. Bars represent mean ± one standard error.

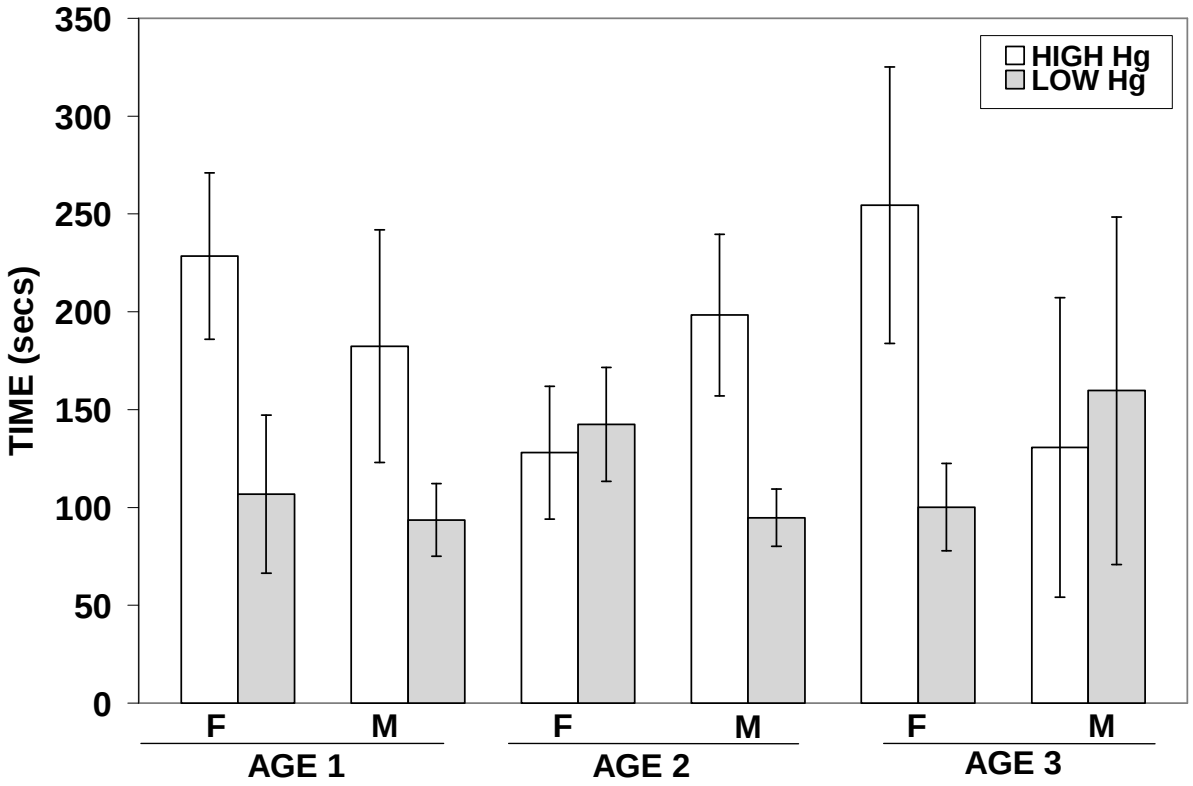

Figure 3.8. Average time to seek shelter by Hg exposure, age and sex. Bars represent mean ± one standard error.

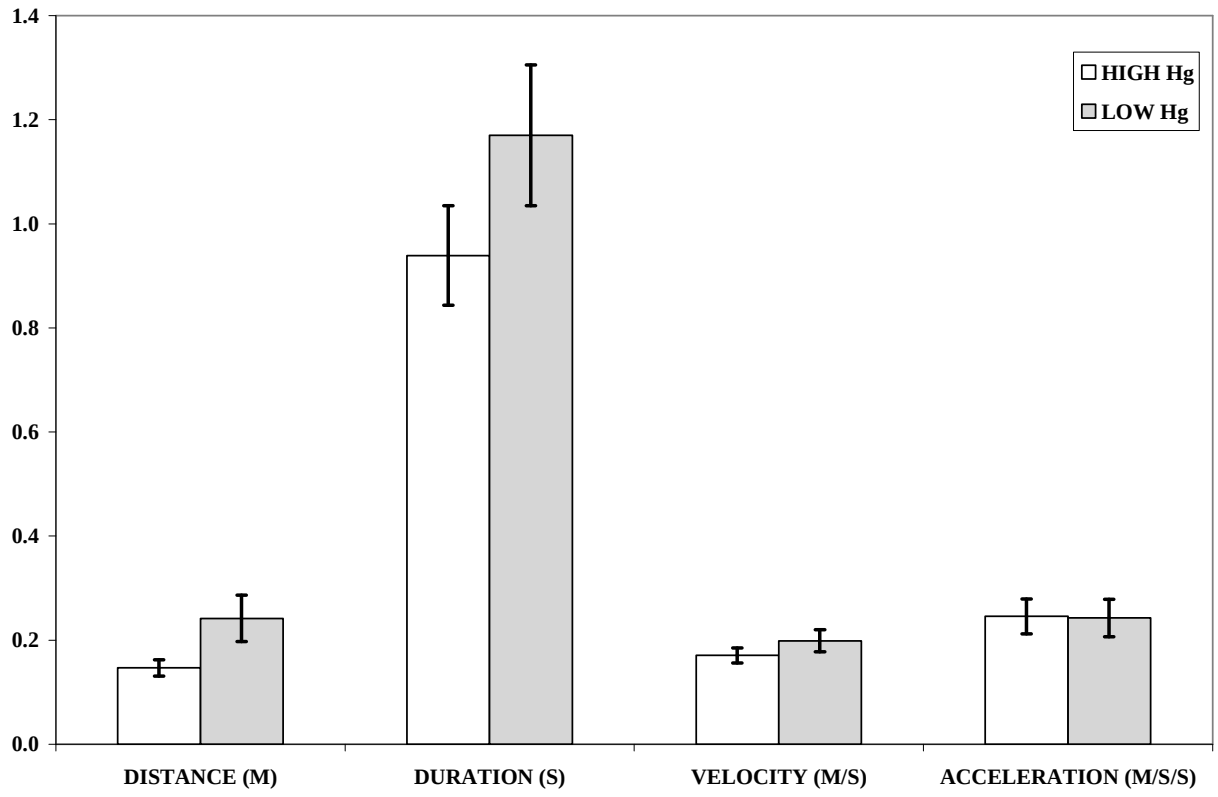

Figure 3.9. Average distance, duration, velocity and acceleration for each Hg exposure. Total distance is measured in meters, total time in seconds. Velocity is defined as the total distance divided by the total time. Acceleration is defined as the velocity divided by the total duration. Bars represent mean $\pm$ one standard error.

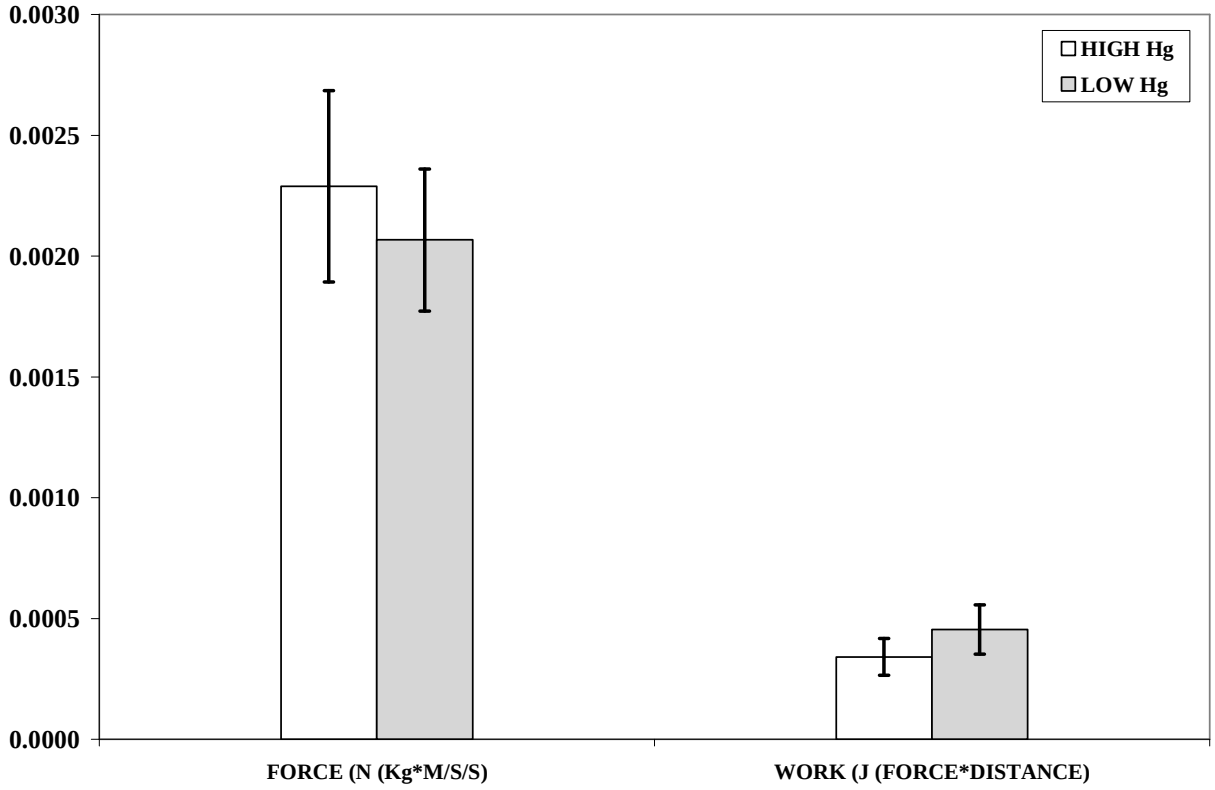

Figure 3.10. Average force and work for each Hg exposure. Force is defined as the weight of the animal in kilograms, multiplied by the acceleration. Work is defined as the force multiplied by the total distance. Bars represent mean $\pm$ one standard error.

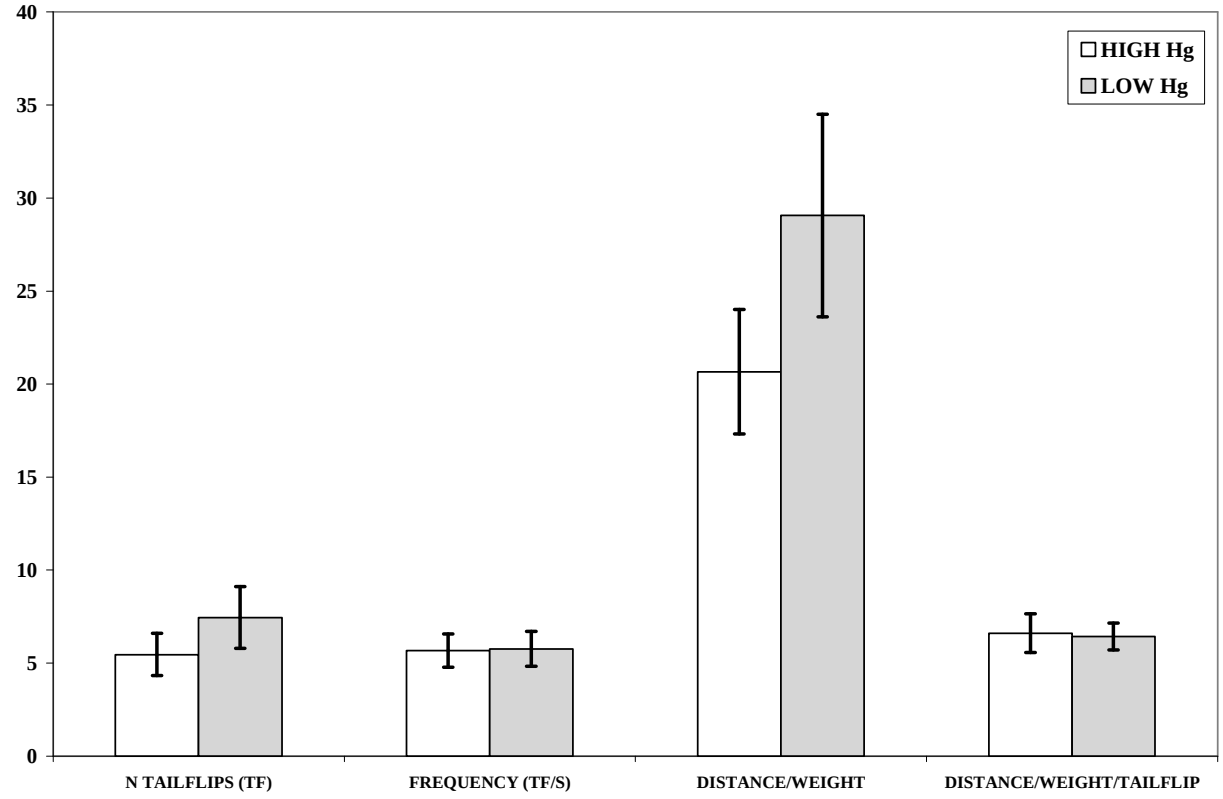

Figure 3.11. Average number of tailflips, frequency of tailflips, distance corrected for weight, and distance by weight by tailflip for each Hg exposure. The tailflip is a direct count of the number of complete flips during the entire escape response. Frequency is defined as the number of tailflips divided by the total time. Distance by weight and distance by weight by tailflip crudely corrects for any variability that are due to the difference in size or weight of the animals. Bars represent mean $\pm$ one standard error.

CHAPTER 4

CONCLUSION

There is sex and age related differences in Hg accumulation when crayfish are chronically exposed to environmentally realistic concentrations in the diet. The younger crayfish from the high Hg exposure accumulated more Hg, with higher mortality than the older crayfish of the same exposure. The females of the high Hg exposure had higher Hg tissue concentrations than the males. However, the relationships of sex and age are not evident in elimination, with suppression of Hg elimination through the molted exoskeleton in both Hg exposures. Major differences seen between the pre-molt and molted exoskeleton of both Hg exposures suggests mobilization and re-absorption of Hg. All of these factors could confound understanding the flux of THg and MMHg into higher trophic positions and therefore should be included within any future studies.

Based on measurement of multiple endpoints, younger male crayfish seem to be most sensitive to Hg exposure. Changes in growth may be a concern for the young males, but also the sexually-maturing (age 3) juvenile males and females. The reduced growth during a time when rapid growth is especially crucial has negative implications not only for an individual, but to the population. Although mercury is a suspect endocrine disrupter, we observed no difference in molting frequencies, a hormonally mediated process. Results suggest that behavior may be a sensitive indicator of mercury toxicity in crayfish. Alterations in each endpoint studied may have negative impacts to the individuals. The summation of these impacts suggests that

environmentally-realistic dietary Hg exposure may have deleterious effects on crayfish populations.