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Interaction between a Low-Iron Diet and Early-Life Methylmercury Exposure in Daphnia pulex

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INTERACTION BETWEEN A LOW-IRON DIET AND EARLY-LIFE METHYLMERCURY EXPOSURE IN *DAPHNIA PULEX*

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ABSTRACT

Methylmercury (MeHg) is a known neurotoxicant and bioaccumulates in fish, with exposure to humans *in utero* being of highest concern. Iron deficiency (ID) accounts for approximately 50% of anemia cases. Anemia is estimated to affect 1.62 billion people worldwide, and is particularly problematic during pregnancy and early life. Previous studies have shown that ID exacerbates toxicity associated with exposure to metals, including lead, manganese, and cadmium. The overall purpose of this thesis research is to investigate the interaction between a low-iron (Fe) diet and early-life exposure to MeHg in *Daphnia pulex*. I hypothesized when *D. pulex* are fed a low-Fe diet, the toxicity associated with early exposure to MeHg will increase. *D. pulex* were fed a standard and low-Fe diet with freshwater algae, *Ankistrodesmus falcatus,* cultured in a standard or low-Fe media. Early-life exposure to MeHg (1600 ng/l) was administered (for 24 hrs in the first 72 hrs. of life), and its effects on lifespan, maturation time, average brood size, and reproduction rate were evaluated. In addition, the effects of low-Fe diet and MeHg exposure on lipid storage were measured using image analysis of Oil Red O (ORO) staining and were also examined through biochemical quantification of total triacylglycerol (TAG). Results suggest that *D. pulex* that are fed a standard Fe diet had no significant difference in lifespan or reproduction (average brood size and reproduction rate) compared to *Daphnia* on a low-Fe diet. Also, there was no difference in maturation time between low and standard-Fe diets of *Daphnia* as well as between MeHg exposed

and vehicle exposed *Daphnia* fed either diets. However, we observed a significant delay when *Daphnia* were exposed to either the vehicle or MeHg compared to the no exposure control. Image analysis for lipids stained with ORO suggests an interactive effect of diet and MeHg exposure, with MeHg exposure increasing lipid storage particularly in *D. pulex* fed a low-Fe diet. Measurement of TAG by using a commercially available kit were not consistent with the ORO results, possibly due to variation introduced from pooled samples and interference from free glycerol in *D. pulex.*

Keywords: metals, iron deficiency, methylmercury, lipid storage, daphnia

DEDICATION

To my parents, Glenda and Emanuel Hudson, for being major supporters of my life.

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TABLE OF CONTENTS

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CDC	Center for Disease Control and Prevention
CVD	cardiovascular disease
EC_{50}	half maximal effective concentration
EDTA	ethylenediaminetetraacetic acid
F_0	parent generation
F_1	first filial/ offspring generation
F_2	second filial/ children of the offspring
Fe	iron
GSH	glutathione
GSH-Px	glutathione peroxidase
Hg	mercury
ID	iron deficiency
IDA	iron deficiency anemia
LC_{25}	lethal concentration for 25% of the population
MeHg	methylmercury
MeOH	methanol
Mn	manganese
NIH	National Institutes of Health
ORO	Oil Red O

SEM	standard error of the mean
TAG	triacylglycerol
WHO	World Health Organization

INTRODUCTION

Iron Deficiency

Iron deficiency (ID) is the most common micronutrient deficiency in the world and is particularly prevalent among women at reproductive age, infants, and young children (CDC 2002). In the US, with the presence of iron (Fe)-fortified foods and high availability of Fe supplements, the prevalence of ID is 7% among toddlers aged 1-2 years and 9-16% among women of reproductive age (CDC 2002). In addition, anemia affected about 1.62 billion people worldwide in 2005, with about half of anemia cases caused by ID, also called iron-deficiency anemia (IDA) (Stoltzfus 2003; WHO 2008). Common risk factors for ID and IDA among adults include a Fe-poor diet, poor absorption of Fe, blood loss due to menstruation, and pregnancy which results in an increased need for Fe (Kalaivani 2009). Furthermore, risk factors or indicators for ID at an early age include premature birth, low birth weight, maternal ID, low dietary intake of Fe, prolonged bottle feeding, and excessive consumption of cow's milk (Parkin & Maquire 2013).

Fe is an essential trace element for almost every living organism, and Fe-containing proteins are involved in enzymatic and antioxidant activities, energy metabolism, carrying and storing oxygen, and transporting and storing Fe (Dallman 1986). In particular, it has been noted that maximal Fe intake in the brain occurs during the rapid period of brain development in rats (Clarkson & Strain 2003).

A complete depletion in Fe storage occurs before the development of IDA (Dallman 1986). ID reduces muscle oxidation capacity, reduces immunity against

infection, and increases cardiac output (Dallman 1986). Most research in ID has analyzed the neurocognitive effects among young children, adolescents, and adults. For instance, IDA has been associated with adverse effects in work productivity, attentiveness, and intolerance to the cold in adults (Friendman et al. 2012). In children, ID has been correlated to impaired binding sites for dopamine and serotonin in the brain, resulting in behavioral changes in children such as increased irritability and becoming uninterested in their surroundings (Dallman 1986). Also, Clarkson and Strain (2003) noted that ID has been associated with adverse effects in mental development among infants and children such as alterations in attention span and decreased intellectual scores; however, ID has not been associated with clinically-defined retardation (Stoltzfus 2003). Moreover, an Icelandic study found no association between Fe depletion and IDA in children at 12 months of age and developmental scores in children at 6 years of age; however, children who were identified with only Fe depletion had significantly lower scores in the described self-help test, a measurement that pertains to the child's ability to take care of oneself, compared to children who were not defined with Fe depletion or were defined with a combination of Fe depletion and IDA (Thorisdottir et al. 2013).

Past research suggests that ID alters the metabolism of toxic substances. For example, it has been shown that ID reduces glutathione peroxidase (GSH-Px) activity per red blood cell in rats as well as reducing the selenium-dependent GSH-Px by 75% in the liver (Lee, Layman, & Bell 1981). The suggested mechanism for this phenomenon is that the reduction of Fe suppresses the protein synthesis of selenium-dependent GSH-Px (Moriarty et al. 1995). Furthermore, the glutathione (GSH) levels significantly decreased among Egyptian children ages 1-12 years (Al-Biltagi et al. 2013) with IDA. Also, the

presence of intestinal cytochrome P-450 is dependent on dietary Fe intake and decreases in Fe deficient rats; however an increase of cytochrome P-450 was observed in the liver (Dallman 1986). Also, past research has suggested that ID decreases catalase activity in the liver of rats (Lee, Layman, & Bell 1981).

Lifespan

ID has been shown to be associated with infant and maternal mortality during childbirth in developing countries (Zimmermann & Hurell 2007; Brabin, Brabin, & Gies 2013). In parallel with this finding, Gambling and other researchers (2003) found that offspring of Fe deficient mother rats had a lower survival rate in the first 8 hours compared to Fe sufficient mothers. However, Haider et al. (2013) found that there was no significant difference in perinatal mortality in anemic mothers compared to non-anemic mothers. Also, a placebo-controlled, randomized study in Nepal found that oral supplementation of Fe and folic acid among children ages 1-36 months had no significant benefit in reducing mortality rates compared to those with a placebo (Tielsch et al. 2006).

Later in life, ID has been associated with cardiovascular disease (CVD). For example, Hsu et al. (2012) found a dose-response relationship between serum Fe levels and CVD risk or mortality among Taiwanese elderly ($\geq$ 65 years of age) in long-term care facilities, with increasing severity of ID increasing the risk of CVD and mortality. Moreover, a European study found that ID is common among patients with chronic heart failure and relates to the severity of the disease; also, an increase of all-cause mortality rates was observed in heart failure patients with ID (Klip et al. 2013).

Reproduction

Past studies have investigated the reproductive effects of ID, predominantly, in terms of preterm birth and low birth weight among humans. For example, Scholl et al. (1992) found that mothers who were Fe deficient had a higher risk of preterm birth (< 37 weeks of gestation) compared to the Fe sufficient mothers. In a Hungarian case-control study, researchers found that there was no significant difference between gestation age and birth weight between anemic and non-anemic mothers who had given birth to infants without congenital anomalies, but they mentioned that Fe supplementation was associated with a slight increase in the gestation period and birth weight, although these increases were not statistically significant (Bánhidy et al. 2011). Moreover, a meta-analysis, conducted by Haider et al. (2013), found that an increase in hemoglobin is associated with an increase in birth weight while anemia in the first and second trimesters is associated with a higher risk of preterm birth and stillbirth. These researchers also found that the use of Fe and folic acid supplementation showed no significant effect on preterm birth; however, Fe and folic acid supplementation is found to increase birth weight in infants, with an increasing dosage of Fe resulting in increased birth weight (Haider et al. 2013). However, Fe-folic acid supplementation or multiple micronutrient therapy showed no significant difference on stillbirth outcomes among pregnant Pakistani women with anemia (Bhutta et al. 2009).

In addition, there is some research that analyzes the effects of ID and fertility in animal models. For example, no significant fertility effects such as differences in pregnancy rate and number of offspring were observed in rats when fed a Fe-restricted diet (Gambling et al. 2002; Gambling 2003). Also, the onset of puberty in female rat

offspring of Fe deficient mothers showed no difference from Fe-sufficient mothers (Gambling 2003). On the other hand, ID from a low-Fe diet was associated with reduced conception rate in rats (Li et al. 2014).

Lipid reserves

There are several studies that observed an association between obesity and ID; however, the relationship between obesity and ID remains unclear. Chung et al. (2011) observed a decrease in hepatic Fe in obese rats fed a high fat diet compared to the controls; from this, they concluded that obesity is a risk factor for ID. Mochonis et al. (2012) reported that obese preadolescents had a significantly higher prevalence of ID compared to overweight and normal weight preadolescents.

In addition, ID has been shown to have effects on lipids in past studies. For example, among Indian adults, those with IDA had a significant increase in triacylglyerol (TAG) levels compared to those who did not (Nandyala et al. 2013). Once these adults were given Fe therapy, researchers observed a significant decrease in TAG levels after 3 months (Nandyala et al. 2013). Stangl and Kirchgessner (1998) found that ID alters the metabolism of lipids in rats and that TAG levels in the livers of Fe deficient rats were not affected while serum TAG levels were increased compared to Fe sufficient rats. Davis et al. (2012) observed an increased amount of TAG in ID rats that were fed a sucrose-based diet compared to rats fed a Fe-sufficient diet and the Fe-deficient rats that were fed a corn starch-based diet. Researchers found that ID up-regulated gene expression responsible for lipid metabolism such as sterol regulatory binding protein-1c and fatty acid synthase in the sucrose-based diet, but no significant increase in expression of these genes were

found in the corn starch-based diet (Davis et al. 2012). Moreover, maternal ID has been found to alter the lipid composition, but not the concentration in the brains of guinea pig offspring as well as decreasing total lipid concentration in the liver (LeBlanc et al. 2009).

Interactions with Other Metals

There has been preliminary evidence that ID may exacerbate the toxicity of other metals. For example, Goyer (1997) noted that there was an inverse relationship between ferritin levels in the blood and cadmium absorption in the gastrointestinal track. Moreover, Peraza et al. (1998) reported that workers who were exposed to cadmium had decreased hematocrit and hemoglobin levels. In a controlled laboratory setting, researchers demonstrated that cadmium interferes with Fe metabolism and absorption, causing a reduction in kidney and liver Fe concentrations in rats (Peraza et al. 1998). To support this finding, cadmium and lead in the blood and urine of anemic Pakistani children (1-10 years) was measured and an association was found between an increased concentration of cadmium and lead and a decrease in blood ferritin levels (Shah et al. 2010). However, a Swedish study, conducted by Berglund et al. (1994), did not find a significant association between blood ferritin levels and cadmium concentration in the blood and urine among nonsmoking women of reproductive age (20-50 years of age) who reported a high-fiber or mixed diet. In addition, a study was conducted in Japan to investigate the absorption of dietary cadmium in Fe deficient, nonsmoking women; from this, researchers found an insignificant increase in cadmium concentrations in the blood and urine in Fe deficient women compared to their controls (Tsukahara et al. 2002). To investigate the mechanism of ID and metals toxicity, Park et al. (2002) found that

divalent metal transporter 1 (DMT1), a protein that primarily transports Fe through cellular membranes, was associated with increased intestinal cadmium absorption in Fe deficient rats after 48 hours of cadmium exposure through oral administration.

Along with cadmium, the absorption and toxicity of other metals has been investigated in Fe deficient subjects. For example, Wright et al. (1999) observed that ID is significantly associated with low-level lead poisoning among children 9 to 48 months. Past research suggested a mechanism that, during absorption in the gastrointestinal track, lead competes with Fe binding sites and inhibits ferrochelatase activity (Goyer 1997; Peraza 1998). However, it is not known if blood-lead levels are increased due to ID or if ID is the result of lead poisoning. Furthermore, Goyer (1997) explains that ID is also responsible for the increased absorption of aluminum. Since aluminum has similar properties as Fe, it has been suggested that aluminum competes with Fe in its specific binding sites in the gut. Also, ID led to an increased absorption of manganese chloride in rats and increased manganese (Mn) toxicity (Chandra 1976). Heilig et al. (2004) confirmed Mn blood levels were 3 to 4-fold higher in Fe-deficient rats; the author later explains that although lung absorption of Mn was not significantly increased by ID, the clearance of Mn in the blood was reduced. One suggested mechanism for Mn toxicity is that trivalent Mn binds to transferrin, a serum protein, and circulates through the blood to the brain, where toxicity occurs (Heilig et al. 2004; Erikson 2005). To our knowledge, the interaction between methylmercury (MeHg) and ID has not been observed.

Methylmercury

MeHg is a known neurotoxicant that affects the developing brain and leads to intellectual and developmental disabilities (Hong 2012). Most MeHg production is due to the release of inorganic mercury (Hg) into the air from natural and anthropogenic sources and is subsequently methylated by aquatic microorganisms (ASTDR 1999). The United Nations Environmental Programme (2013) estimates that 30% of Hg emissions is due to anthropogenic sources; in which, small artesian gold mining is responsible for the majority of the anthropogenic releases at more than 35% while coal burning is the second man-made release of Hg at more than 20%. Moreover, about 10% of the total Hg emissions come from natural emissions such as volcanic activity, while 60% is due to re-emission of the Hg that already exists in the environment. Released elemental and inorganic Hg deposits in water bodies, the microorganisms of the aquatic system methylate Hg; thus, increasing its bioavailability to other organisms and bioaccumulating in fish (Gochfeld 2003).

The primary source of human exposure to MeHg is through fish consumption, with an increased risk from consumption of fish from higher trophic levels such as shark, marlin, and aquatic mammals (ASTDR 1999). In addition, infants also acquire MeHg prenatally via *in utero* exposure or through the consumption of contaminated breast milk (Bakir et al. 1973). The clinical manifestations of acute MeHg poisoning in adults includes a tingling sensation around the mouth, hands and feet; muscle weakness; and trouble with coordination (Clarkson, Magos, & Myers 2003; Taber & Hurley 2008). Severe poisoning symptoms include blindness, coma, and death among adults (Bakir et al. 1973). Furthermore, prenatal exposure to high-level MeHg results in cerebral palsy-

like symptoms in young children such as severe retardation, deformity of the limbs, improper growth and nutrition, and microcephaly even when the mothers were asymptomatic (Harada 1978).

MeHg became the center of public health concern since the industrial mercury pollution in the waters around the Minamata area of Japan in the 1950s and 1960s, causing widespread MeHg poisoning, also diagnosed as Minamata disease, among villagers who consumed the highly contaminated seafood (Harada 1978). Symptoms of Minamata disease in adults included but were not limited to impairment of speech, hearing impairment, tremors, and reduced visual fields (Eto 1997). Although expectant mothers of the region showed no signs of Minamata disease, their children showed apparent signs of MeHg poisoning (Harada 1978; Clarkson & Strain 2003).

Another widespread MeHg poisoning occurred in Iraq in 1971-72 and was responsible for over 450 deaths (Bakir et al. 1973; Al-Damluji & CCMP 1976). The Iraqi MeHg poisoning epidemic was the result of the improper use of seed grain that was treated with antifungal MeHg for homemade bread making in rural areas (1973; 1976). Iraqis suffering from MeHg poisoning showed similar symptoms as Minamata disease, and from this, researchers found that the severity of MeHg poisoning symptoms is dose-dependent and vulnerability is age-dependent (Al-Damluji & CCMP 1976; Hurley, Hayman & Taber 2008). Also, they found that MeHg has a latent effect of its toxicity, with symptoms appearing on an average of 3 weeks after exposure (Al-Damluji & CCMP 1976). Through additional epidemiologic research, the Iraqi MeHg poisoning confirmed that the fetus is more susceptible to MeHg exposure compared to adults (Harada 1978).

Mechanism of MeHg Toxicity

MeHg and its toxic effects have been studied at the molecular level as well as the cellular and organ pathological level; however, the mechanism of MeHg toxicity is not yet fully understood. This organic compound is lipid soluble and absorbs easily in the lungs, on the skin, and in the gastrointestinal tract with a half-life of about 50 days in humans (Peraza et al. 1998; Gochfeld 2003). Moreover, it is well known that MeHg has a high affinity to sulfhydryl (thiol) groups, making multiple targets available for toxicity such as cysteine and mimics methionine to cross the blood-brain barrier and placenta to affect the brain, as well as binding to other thiol-containing proteins, resulting in nonspecific cell injury or death (Hurley, Hayman, & Taber 2008; Liu, Goyer, & Waalkes 2008; Peraza et al. 1998; Liu, Goyer, & Waalkes 2008).

Also, past studies indicated that MeHg causes oxidative stress, lipid peroxidation and neuronal cell damage as well as damage to microtubules in cells (Peraza et al. 1998). In common Loon (*Gavia immer*) chicks, Kenow et al. (2008) found when *G. immer* were exposed to dietary MeHg at 0.4 and 1.2 µg Hg/g, increasing blood Hg was associated with an increased amount of oxidized glutathione, a sign of oxidative stress; this finding was also paired with the reduction of GSH-Px activity in the brain of the 105 day old chicks.

Low-level MeHg Toxicity

At low levels, MeHg exposure significantly reduced the number of neuronal stem cells in rats as well as an observed reduction of neurons in the dentate gyrus of the hippocampus (Bose et al. 2012). This exposure did not cause apoptosis or necrosis in

neuronal stem cells from rats; however, it altered the cell cycle regulators, lengthening the cell cycle and thereby reducing the total number of cells (Bose et al. 2012).

Later, Guo et al. (2013) showed prenatal exposure to MeHg (0-1mg/kg/day) is associated with disruption of the neuronal migration in the cerebral cortex of offspring rats. Similar to Bose et al., they found that MeHg did not induce apoptosis; however, it did alter the proliferation of neural progenitor cells (Guo et al. 2013).

MeHg and lifespan

Past studies have suggested that MeHg exposure does not affect lifespan. From a life table analysis study, exposure to MeHg did not affect the life expectancy of the Japanese (Tamashiro, Fukutomi, & Lee 1987). Also, MeHg did not affect the survival of adult fathead minnows (Hammerschmidt et al. 2002). Lifespan was not affected in mice when given 1ppm of MeHgCl administered in the water; however, among mice that were administered 5ppm in water and then were administered 1ppm later in life (> 90 days of age), the survivors had a longer lifespan compared to those given a lower dose early in life (Schroeder & Mitchener 1974). In addition, Helmcke et al. (2009) did not observe an effect in lifespan of surviving nematodes (*Caenorhabitis elegans*) when exposed to water-dissolved MeHgCl for 30 minutes (0-1.5 mM MeHgCl) or 15 hours (0-0.4 mM MeHgCl). Furthermore, a significant decrease in the survival of male *Fundulus heteroclitus*, a forage fish from the marine ecosystem was observed after dietary exposure to MeHgCl (0.5 -11 µg MeHg/g) compared to their gender-paired controls (Matta et al. 2001).

Reproduction effects

Past studies have reported that low levels of MeHg did not affect conception or live births in women; however, at high levels, MeHg drastically reduces conception rate (Harada 1968; Zahir et al. 2005). Moreover, primate studies, summarized by Mottet et al. (1985), have shown a relationship between increasing maternal blood MeHg (< 1.0 ppm) and increasing rate in reproductive failures (i.e., decreasing pregnancy rate as well as an increase in abortion rate). However, low doses of MeHg have been suggested to increase reproduction in birds. The percentages of the hatched eggs and number of 6 day-old mallard (*Anas platyrhynchos*) ducklings were significantly higher in those that were fed a daily commercial duck diet mixed with 0.5µg/g $MeHgCl_2$ dissolved corn oil versus the duck diet alone(Heinz et al. 2010). However, higher doses of MeHg (>4.6 mg/kg dry weight) had a negative effect on eggs hatched and number of fledglings among American Kestrels (*Falco sparverius*) (Albers et al. 2007). Along with reproduction in birds, brood size significantly decreased in *C. elegans* when exposed at a concentration of 1mM MeHgCl at early life; however, other concentrations of MeHg (0.1, 0.4, & 10 mM) did not show an effect on brood size (Helmcke et al. 2009). In another study, a decrease in *C. elegans* brood size is observed in chronic MeHg exposure (VanDuyn et al. 2010). In fish, dietary MeHg has been shown to reduce the number of eggs laid and spawning success of fathead minnows; dietary MeHg exposure also reduced the gonadal development in female fathead minnows (Hammerschmidt et al. 2002). Furthermore, MeHg caused altered reproductive behaviors of male fathead minnows (Sandheinrich & Miller 2005). One study investigating the reproductive effects of MeHg in *F. heteroclitus* found that fecundity was not significantly altered after dietary exposure to MeHgCl

compared to the controls; however, a non significant decrease in fecundity was observed after exposure to the highest concentration (11 µg/g) tested (Matta et al. 2001).

Lipid reserves

There have been some studies that investigated the effects of Hg-containing compounds in lipid reserves. Drevnick et al. (2008) observed that lipid reserves decreased when exposed to high inorganic Hg concentrations and an increased accumulation of lipofuscin, a dark-colored pigment that indicates lipid peroxidation, in the liver of Northern Pike. Moreover, Kawakami et al. (2011) suggested that mice on a high-fat diet showed a significant reduction in white adipose tissue, as well as a significant reduction of plasma TAG, after exposed to subcutaneous injections of mercuric chloride (HgCl) for 10 days. Moreover, the rats' TAG levels in the brain, spinal cord, liver, and kidney had reduced significantly when exposed to a daily dose of MeHg at a concentration of 1 mg/kg for 7 days (Sood et al. 1997). In another study, dietary exposure of MeHg (1-2 ppm) in rats that were fed a 5% fish oil diet showed a significant decrease in plasma TAG concentration compared to control rats and rats on a 5% lard diet; however, when rats were fed a lard diet and exposed to 2ppm MeHg, a significant decrease in phospholipids was observed and not in the fish oil diet (Shirai et al. 2012).

Interaction with Nutrition

Many epidemiological and experimental studies have investigated the association between MeHg exposures and nutrition. The most famous epidemiological research studies for MeHg exposure were the Seychelles (Davidson et al. 1998), Faroe Islands

(Grandjean et al. 1997; Debes et al. 2006), and New Zealand (Kjellström et al. 1986; Kjellström et al. 1989). In the Seychelles study, 711 mother-child pairs were investigated to determine associations between prenatal and postnatal exposure to MeHg, primarily through the consumption of ocean fish, and neurodevelopment of children through a series of evaluations. No association between neurodevelopmental outcomes and MeHg exposures was found in this cohort (Davidson et al. 1998). On the other hand, the primary exposure to MeHg in the Faroe Islands is due to the consumption of whale meat. Among those who were prenatally exposed to low levels of MeHg, an adverse effect in language abilities, memory, and attention was observed in children at 7-years old (Grandjean et al. 1997). Later, by 14 years of age, researchers found a correlation of prenatal MeHg exposure and adverse effects on attention and language abilities as well; however, they found a negative association in motor speed, not in memory (Debes et al. 2006). Therefore, it is believed that the dietary source of MeHg influences its toxicity.

Also, deficiencies in trace essential elements have been shown to alter the toxicity of MeHg, especially in selenium and zinc (Chapman & Chan 2000). Selenium has been shown to decrease the toxicity of MeHg. For example, Bjerregaard and Christensen (2012) found an increase in MeHg elimination when fed selenium in brown shrimp, *Crangon crangon*. Furthermore, this study also noted that the MeHg-selenium interaction was dependent upon exposure time, concentration, and type of selenium compound such as, selenite, Se-cystine, and Se-methionine enhance MeHg elimination in *C. crangon* but, selenate did not (Bjerregaard & Christensen 2012). Also, Beyrouty and Chan (2005) found no protective effect of selenium supplements in terms of postnatal survival among MeHg exposed rats; however, a protective effect in postnatal survival

was observed when selenium was administered in conjunction with vitamin E. In addition, a recent study suggested that Fe status is not associated with MeHg levels among Amazonian women of reproductive age (12-49 years); therefore, Fe status, whether Fe deficient or not, did not alter MeHg concentrations in the hair (Fonesca, et al. 2014).

Daphnia as a model organism

For this study, the model *Daphnia pulex* was selected for toxicity testing of early-life MeHg exposure. *D. pulex*, a freshwater crustacean, is listed as a National Institutes of Health (NIH) model organism for biomedical research. It is widely used for ecotoxiological studies and is believed to be a sensitive model to investigate developmental and disease processes (NIH 2013). Lampert (2006) notes that *D. pulex* is easy to maintain in laboratory cultures, due to its quick reproduction and short generation times of about 60 days; therefore, a large population can be produced within a short period.

Due to its phenotypic and ecological diversity, *D. pulex* is suggested as a versatile model to investigate macromolecular relationships and cellular functions (NIH 2013). Moreover, it is an ideal model for toxicity testing due to its quick responses to environmental changes and to its transparency for physiological studies such as pathogens, parasites, respiration, osmoregulation, food uptake, digestion, and growth patterns (NIH 2013; Lampert 2006). There is some evidence that mineral limitation by the imbalance of chemicals affects *Daphnia's* growth while population crowding, morphology and biochemical composition affects the performance of *Daphnia* (Lampert

2006). Also, since it carries developing eggs in its brood pouch, calculating *D. pulex* life history and population dynamic parameters is relatively easy (Lampert 2006).

In addition, the organism's energy and carbon budgets were constructed from physiological data to estimate its production under various conditions, leading to the sophisticated mathematical models that describe *D. pulex* growth processes, reproduction, and survival (Lampert 1977; Lampert 2006). Since the organism's genome, physiology, population dynamics and ecosystem impact have been well-studied, *D.pulex* is also accessible for mathematical modeling in various environments (Lampert 2006). With the recent sequencing of the *D. pulex* genome, it could be used as a model to investigate the interaction between environmental stressors and genes (NIH 2013). Furthermore, this model can reproduce parthenogenetically (clonal reproduction); therefore, clones are able to be maintained in laboratories for an extended period of time (Lampert 2006). Also, researchers can analyze tissue-specific gene expression at any life stage due to its transparency. Moreover, *Daphnia* reproductive effects are sensitive to food quality, in particular, to low nitrogen concentrations of *Ankistrodesmus falcatus* (Kilham 1997).

Lipid Storage

Lipid storage in *D. pulex* is mostly composed of TAG and indicates its feeding success (Tessier & Goulden 1982). These lipids, inherited from the mother to the neonates, offer protection against starvation and fluctuate according to population size (Tessier & Goulden 1982; Goulden & Hornig 1979). Goulden and Price (1990) also found that most lipids (>98%) in *Daphnia* originate from the organism's diet. This

means that *Daphnia* is highly dependent on the quality of their diet since a minute amount of lipids are synthesized. Also, reproductive success can be estimated by lipid storage (Tessier & Goulden 1982), with an increased presence of lipids indicating a higher success in reproduction; furthermore, this study observed a positive relationship between body size and lipid storage size, except in neonates and *D. pulex* juveniles at the late instar (Tessier & Goulden 1982). A past study concluded that lipid reserves in *Daphnia* are responsive to stressors such as temperatures, extreme light, and selective predation (Filho, Soares, & Loureiro 2010). In particular, TAG in *D. pulex* fluctuates in different seasons and is influenced by temperature, with the highest concentrations observed in the cooler fall and winter months (Arts et al. 1992).

Along with physical environmental factors, TAG in *Daphnia* is also responsive to chemical exposures. In particular, lipids of *D. magna* are considered as the most sensitive of all energy reserves after acute exposures to chemical stressors (DeCoen & Janssen 2002). Arzate-Cárdenas and Martínez-Jerónimo (2012) found that energy reserves, consisting of carbohydrates, lipids, and proteins, in *Daphnia* are reliable stressor biomarkers to evaluate the effects of hexavalent chromium toxicity at sublethal doses ($1/5^{th}$ and $1/25^{th}$ of EC_{50}). From this, they observed a decrease in lipid reserves when *D.schodeleri* were exposed to hexavalent chromium except among 5 and 7 day-old organisms; also, 3 and 28-day-old *Daphnia* did not exhibit a decrease in lipids when exposed to hexavalent chromium at 1/25 of EC_{50} (6.3µg/L and 10.4µg/L for their respective ages) (Arzate-Cárdenas and Martínez-Jerónimo 2012).

Iron in D. pulex

In *D. pulex*, Fe is required for hemoglobin synthesis, which assists *Daphnia* with survival in low-oxygen environments (Smaridge 1956; Engle 1985). During hemoglobin synthesis, Fe can be found mostly in the gut walls, fat cells, and ovaries; when the *Daphnia* is moved from a hypoxic environment to an aerated environment, hemoglobin is no longer needed and the Fe is excreted through the shell-glands (Smaridge 1956).

Past studies have investigated the presence of Fe and its toxicity in *Daphnia*. For example, the 48-hr EC_{50} of supplemental Fe was estimated to be 73.2±17.4 µg/L in the water for *D. magna* (Bošnir et al. 2013). Also, Dave (1984) noted that Fe is essential for reproduction in *Daphnia*. Also, compared to the controls (0 µg Fe/L), he observed reproduction decreased when waterborne concentrations were above 32µg Fe/L and a steady decline in survival at concentrations above 512 µg Fe/L (Dave 1984). However, to our knowledge, there have been no studies that have investigated the effects of ID in *Daphnia*.

Mercury Exposure

There have been several studies that investigated mercury's toxicity in *Daphnia*. For example, Tian-yi and McNaught (1992) analyzed the toxicity of chronic MeHg exposure among *D. pulex* cultured in lake water and its acute and chronic effects on reproduction, growth rate, and natality. This study observed a reduction in age-specific birth rate and total offspring when exposed between 10 ng/L to 1000 ng/L MeHg every 3 days throughout lifespan as well as a gradual increase in early deaths (Tian-yi & McNaught 1992). From this, they calculated the half maximal effective concentration

(EC_{50}) for *D. pulex*'s reproduction in the first generation was 707ng/L while the lethal concentration for 25% of the population (LC_{25}) is 794ng/L (Tian-yi & McNaught 1992). Previous research has suggested that the accumulation of MeHg in zooplankton increases by increasing body size and a higher concentration of MeHg is found in the phospholipids compared to TAGs. However, the same study concluded that MeHg accumulation in these organisms was not affected by dietary intake, but by its habitat (Kainz, et al. 2001). Furthermore, *Daphnia* eliminates MeHg quite rapidly with most of the MeHg loss through water excretion and reproduction (Tsui & Wang 2004). In addition, maternal transfer of MeHg has been observed in the undeveloped eggs and neonates of *Daphnia magna,* and has been associated with increasing MeHg content in eggs as well as a reduced survival rate in the offspring (Tsui & Wang 2004). Tsui and Wang (2004) also found that the maternal transfer of Hg showed a tolerance to Hg in the offspring (F_1) of exposed *D. magna* parents (F_0); however, the F_2 generation did not show a tolerance to Hg. Also, they have observed hormesis, or a beneficial relationship, in the F_1 generation in terms of reproduction when exposed to 1.5nM Hg (Tsui & Wang 2004).

SPECIFIC AIMS

The overall purpose of this study is to investigate the long-term effects of the interaction between poor nutrition, particularly a low Fe diet, and early-life, low-level, exposure to MeHg. I hypothesize when *D. pulex* are fed a low-Fe diet, the toxicity associated with early exposure to MeHg will become exacerbated. To test these hypotheses, the following specific aims have been considered:

(1) Compare the reproductive and life history parameter effects in *D. pulex* that are fed a standard and low-Fe diet and exposed to MeHg early in life. This will be done by analyzing the survival rate, time until first reproduction, and average brood size.

Hypothesis 1: A shortened lifespan will be observed in *D. pulex* when fed a low Fe diet or exposed to MeHg;

Hypothesis 2: *D. pulex* will yield less offspring when exposed to MeHg or when fed a low-Fe diet;

(2) Compare the effects of low-Fe diet and MeHg exposure in *D. pulex* on lipid storage. This will be done by quantifying the amount and size of lipid droplets through image analysis of *D. pulex* stained with Oil Red O (ORO). Lipid levels will also be biochemically examined through fluorometric measurement of TAG levels.

Hypothesis 1: *D. pulex* that are fed a low-Fe diet will have a higher amount of lipids compared to those fed a standard-Fe diet

Hypothesis 2: *D. pulex* exposed to MeHg will have a lower lipid content compared to those not exposed to MeHg.

MATERIALS AND METHODS

Model Organism

The *Daphnia pulex* culture was established from clones sent from Dr. Joe Shaw's laboratory at Indiana University. This clonal line has been maintained in our laboratory since 2011. *Daphnia* were maintained in COMBO media as described by Kilham, et al. (1998) at a density of approximately 20 animals per 1L beaker. COMBO is reconstituted water that has been shown to support *Daphnia* growth and reproduction (Kilham et al. 1998). Briefly, major stocks Calcium Chloride ($CaCl_2$), Magnesium Sulfate ($MgSO_4$), Sodium Bicarbonate ($NaHCO_3$), Sodium Metasilicate (Na_2SiO_3), Potassium Chloride (KCl), and Boric Acid (H_3BO_3) were added along with Selenium and Animate stock solutions to ultrapure deionized water (Elga: Purelab Ultra MK2, TOC 3-10X) and aerated for at least 11 hrs prior to use. *Daphnia* were kept in a temperature controlled chamber ($22.5^{o}C \pm 0.5°C$, Percival, I36NLXC8) on a 12:12 light: dark cycle. Similar to previous research (Shaw et al. 2007), cultures were fed freshwater phytoplankton, *Ankistrodesmus falcatus*, every 48 hours, at a concentration of 80,000 cells/mL, unless as explained below.

Full and Reduced Iron Algae Culture

Ankistrodesmus falcatus algal cells were obtained from Carolina Biological (15-1955 *Ankistrodesmus* Alga-Gro freshwater). Standard-Fe algae were cultured in growth

medium containing Woods Hole MBA Macro- and Micronutrient stocks that includes $CaCl_2$, $MgSO_4$, $NaHCO_3$, Na_2SiO_3, Potassium Phosphate (K_2HPO_4), Sodium Nitrate ($NaNO_3$), ethylenediaminetetraacetic acid (EDTA), Ferrous Chloride ($FeCl_3$), Copper Sulfate ($CuSO_4$), Zinc Sulfate ($ZnSO_4$) Cobalt Chloride ($CoCl_2$), Manganese Chloride ($MnCl_2$), and Ammonium Molybdate ($Mo_7O_{24}(NH_4)_6$) and vitamin stocks (biotin, thiamin, pyridoxine, pyridoxamine, calcium panthothenate, B_{12}, nicotinic acid, nicotinamide, folic acid, riboflavin, and inositol) as detailed by Kilham et al. (1998). To reduce the Fe content in algae, algae were cultured by the stated protocol; however, the $FeCl_2$ stock solution and the Fe chelating agent, EDTA concentrations were reduced by half in the growth medium. Algal cells were harvested via centrifugation and/or filtration and washed with COMBO 3 times to remove any residual $FeCl_2$ and EDTA from the algal growth media.

To determine the feeding rate, a standard curve was developed relating cell number to intensity readings at 680nm wavelength on a Beckman Coulter, DU ® 800 spectrophotometer. First, the concentrated and washed algal cells from each treatment group were diluted (factors of 12, 24, 48, and 96) and counted using a hemocytometer. Diluted algae were also placed in the spectrophotometer to find the optical density. Linear regression was used to estimate the corresponding cell concentration from the spectrophotometer reading. Cell concentrations for each diet were estimated separately since Fe concentrations has been shown to affect chlorophyll content (Kosakowska et al. 2004; Fan et al. 2014). Algal cell size, also shown to alter according to Fe levels (Marchetti & Cassar 2009), was examined in the low Fe versus standard Fe media via microscopy and image analysis using ImageJ (Figure 1). The feed rate was adjusted to

account for a 10% reduction in cell size of *A. falcatus* cells grown in the reduced Fe media, summarized in Table 1.

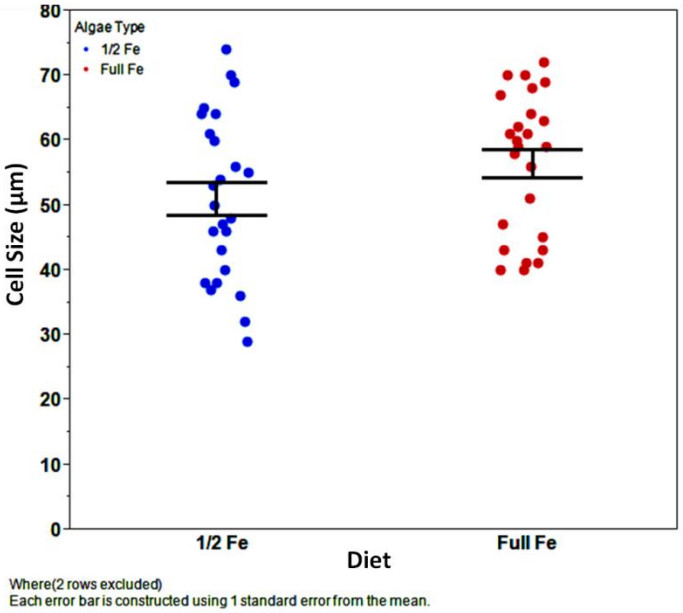

Figure 1. *A. falcatus* Cellular Size Grown in either Standard Media (Full Fe) or Media with the Addition of 50% of the Iron in the Standard Media (1/2 Fe). Error bars represent the standard error of the mean (SEM)

Table 1. Summary analysis of *A. falcatus* Cellular Size

Treatment	**N=**	**Mean (µm) ± SEM**	
Full Fe	25	56.40 ± 2.18	*p*= 0.1124
½ Fe	25	51.00 ± 2.53	

Iron Quantification

Iron content was determined in *A. falcatus* grown in reduced and standard Fe media and *Daphnia* fed *A. falcatus* grown in either standard or reduced Fe media. Fe was quantified using an acid-based commercial assay (QuantiChrom^{TM} Iron Assay Kit BioAssay Systems DIFE-250, Hayward CA). In this assay, hydrochloric acid separates iron from proteins while ascorbic acid reduces Fe^{3+} in the sample to Fe^{2+}; this kit also utilizes a chromogen (tris(2-pyridyl)-s-triazine) to form a blue-colored complex with Fe^{2+}

(Fischer & Price 1962; Schilt & Taylor 1970). Both algal and *Daphnia* samples were sonicated (Branson Sonifier 250) with 10 bursts at a pressure of 30%. The sonicated mixture was incubated with reaction mix at room temperature for 40 minutes and then centrifuged at max speed (13,000 RPM/*g*) for 10 minutes. *Daphnia* of various ages (14-51 days) went through a 24h fasting period before harvested to determine Fe levels to minimize the effect of algae in gut. The fasted *Daphnia* were homogenized with the reaction mix. The homogenized samples were centrifuged and incubated as previously described. 200µL-50µL were loaded into wells of a clear bottom plate, filling with deionized Ultrapure (PURELAB, Elga LabWater) water to reach 250µL. Standards and samples were read by a colorimetric plate reader (Spectramax Plus 384) at peak absorbance of 590nm. The final concentrations for algae and *Daphnia* were expressed in mg Fe/100,000 cells and µg Fe/ *Daphnia*, adjusting for cell count and number of animals, respectively (Garcia et al. 2007).

Methylmercury Exposure

For the stock solution, 10mg of methylmercury II chloride (Sigma Aldrich) were dissolved in 1.7 mL of methanol and then added to 1L deionized Ultrapure water. For each diet treatment, *D. pulex* neonates (≤ 2days) used for experiments were exposed to MeHg at a concentration of 1600ng/L (38.4µL of $MeHgCl_2$ stock solution in 240mL of COMBO media in glass bottles) for 24hrs without food. To verify the effects were due to only MeHg, the treatment groups were paired with a 0ng/L control and a vehicle control (0.2µL/L MeOH). A total of six treatment groups were evaluated: low -Fe diet and early-life MeHg exposure, low-Fe diet and vehicle control, low-Fe diet no exposure control,

standard Fe diet and early-life MeHg exposure, standard Fe diet and vehicle control, and standard Fe diet no exposure control.

Lifespan & Reproduction

Prior to experiments, *Daphnia* were acclimated to either low or standard Fe diets for at least two generations. The parents of experimental animals were individually kept in 50mL polypropylene centrifuge tubes with 30mL of COMBO and checked every 48h for offspring. All offspring after the third clutch were used for experiments. The day the *Daphnia* are harvested from the parents are labeled as "Day 0". Following the 24 hr. exposure period (Day 1), at least 20 *D. pulex* were placed in 50mL centrifuge tubes with 30mL COMBO media, fed with either standard or reduced Fe *A. falcatus* at the respective feed rate described above. *Daphnia* received media changes with fresh algae and were checked for death and number of offspring every 48 hours, starting at "Day 3". Since no exposure controls did not undergo an exposure period, *Daphnia* lifespan and reproduction were checked and recorded, starting at "Day 2". Age at first reproduction, average brood size, total reproduction and lifespan was calculated for each *Daphnia*. Average brood size was calculated by dividing the total number of offspring over the total reproduction cycles while lifespan reproduction was total offspring divided by total days lived.

Oil Red O (ORO) Staining

ORO is a lipid soluble dye that specifically stains neutral lipids and cholesterol esters (Raírez-Zacarías, Castro-Muñozledo, & Kuri-Harcuch, 1992). Modeling from Yen et. al (2010) and Soukas et al. (2009), a stock solution was prepared by adding 0.5g of

ORO powder (Sigma Aldrich) to 100mL of 2-propanol (isopropanol) alcohol and allowed to equilibrate at room temperature for several days. Within 15 minutes prior to staining, the stock solution was diluted with 40% of Ultrapure water and set at room temperature for 10 minutes and then was filtered using a 100µm nylon mesh strainer (Fisher). One part of the diluted stock was mixed with 3 parts of 2-propanol to create a working stock that remains stable for about 2 hours.

Using live, 6-8 day old *D. pulex*, samples were placed in microcentrifuge tubes and water was removed by using pipettes. 1mL of the ORO working stock was added into the tubes, and the closed tubes were inverted several times to resuspend *Daphnia*. The samples were incubated in the ORO working solution at room temperature for 45 to 60 minutes. The stained specimens were then washed with 70% ethanol twice and were fixed using 70% ethanol on a concave microscope slide (Pearl 7103). Fixed *D. pulex* were placed under a microscope (Olympus BX41) at 4x magnification. Using an external light source, light was placed on the bottom outer corners of the slide at full light intensity. The slide was adjusted until the compound eye and lipid droplets were visible and defined. All images were taken with a digital camera (Olympus Q Color 5), using QCapture software.

Image Analysis

Image analysis was conducted using ImageJ freeware (Rasband 2014). To visualize the droplets easily, the dark background was subtracted (Figure 2B). The thresholding method was set on default while the threshold color was set on red. Also, the color space was selected as HSB (Hue, Saturation, and Brightness). To highlight the

red color, the "pass" option was unchecked on the hue selection to activate the band-reject filter while the "pass" option was checked to activate band-pass filter for both saturation and brightness selections. Using the original corresponding image as a reference (Figure 2A), the images were adjusted according to color threshold, highlighting only the red lipid signal, shown in Figure 2C (Deustch et al. 2014). The image was then converted to a grayscale image using the red, green, and blue stack. The green channel was selected (Figure 2D) and thresholded (Figure2E). As described in Stamps and Linit (1995), percentage lipid area is used to measure lipid content of *D. pulex*. To measure lipid area percentage, *D. pulex* was selected around the outer edges of the body, omitting the appendages with use of the freehand selection tool. The selected image was then analyzed by using *Analyze Particles* and summarized. Percentage lipid area was recorded as % *Area* and then compared among diet and exposure groups.

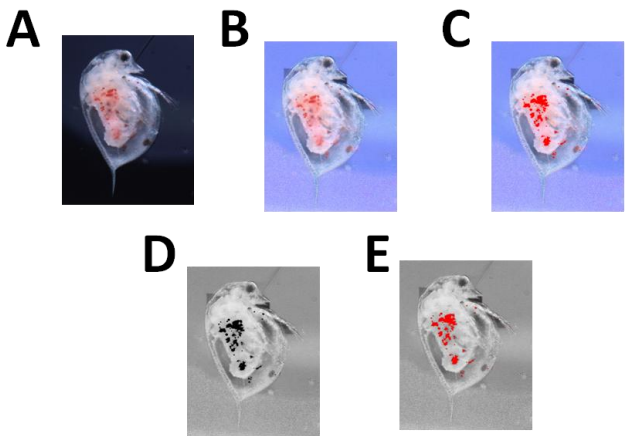

Figure 2. Step-by-step Procedure to Image Analysis for Lipid Staining with Oil Red O in *D. pulex* using ImageJ: (A) Original image (B) Image with dark background subtracted (C) Dyed oil droplets highlighted via threshold, in bright red (D) Green channel selected in the RGB stack (E) Image re-thresholded in red.

Triacylglycerol (TAG) Assay

Lipid content was also evaluated using a commercial TAG fluorometric assay (BioVision, K622-100). This enzymatic kit uses lipase to convert TAG into free fatty acids and glycerol. For every plate, a TAG standard was placed in the wells at a concentration of 0.1, 0.08, 0.06, 0.04, 0.02, and 0 nM to create a standard curve. 6-8 day old *D. pulex* ($n \approx 40$) were placed in 1.5mL microcentrifuge tubes, and wet weight was recorded prior to sample preparation.

Sample preparations and assays were followed as directed on the kit's protocol: *D.pulex* were sonicated in 1 mL of NP-40 (Branson Sonifier 250) and then heated between 85-100^{o}C and cooled to room temperature twice to dissolve the lipids into the detergent. Samples were then centrifuged at max speed to separate any insoluble materials and 10-50µL of supernatant was placed in a black 384 well plate in 10µL intervals for two columns; in wells containing less than 50µL of sample, the TAG buffer filled the wells to reach 50µL. To measure glycerol background, 2µL of TAG buffer only was added to one column of samples; whereas, 2µL of lipase was added to the other column and the standard column. The plate was incubated at room temperature for 20 minutes to convert TAG to glycerol and fatty acids. After incubation, 50µL of the reaction mix, containing a buffer, enzyme mix and a glycerol probe, were placed in wells, covered, and incubated at room temperature for 40 minutes. The prepared plate was analyzed in a fluorometric plate reader (Spectramax Gemini XPS) at excitation/emission phase of 535/590nm. Standard curve and dilutions were analyzed in Microsoft Excel, with the glycerol backgrounds subtracted for the samples, and were adjusted by weight. TAG concentrations were averaged in each group and expressed as µg TAG/animal.

Statistical Analysis

Acute mortality was compared across treatments using the Chi-square test. The survival rate and maturation time was compared across treatments using the Kaplan-Meir distribution. Differences in survival across treatments were determined using the Wilcoxon rank sum test. All missing samples and accidental deaths were censored from the lifespan analysis. Moreover, Fe concentrations, reproductive parameters (total reproduction/reproduction and average brood size), image analysis of lipid/body percentage, and TAG concentrations are compared across treatments using one-way ANOVA. A multivariate linear regression model was used to estimate the modifying effects of diet, total body area, amount of *Daphnia* in jars (Jars N), exposure to MeHg and exposure to MeOH (independent variables) on lipid storage (dependent variable). Analyses were conducted in JMP 10 and SAS 9.3. If there was no significant difference between the vehicle control and no exposure control group in each respective diet, the two groups were combined. All statistics were deemed statistically significant when $P \leq 0.05$.

RESULTS

Iron Quantification

Algae (*A. falcatus)*

A reduced Fe diet for *Daphnia* was produced by culturing *A. falcatus* in standard (3.15mg $FeCl_3$/L) and low Fe media (1.57mg $FeCl_3$/L). After harvesting, the Fe content of *A. falcatus* grown in standard or reduced Fe media was measured. The mean Fe concentration of *A. falcatus* grown in the standard Fe growth media contains more than 5-fold the amount measured in media containing low Fe media (Table 2.). The mean Fe concentration in Full Fe is significantly higher than the algae grown in half Fe media (t= 0.0480). As a result, it is appropriate to label *A. falcatus* grown in low Fe media as "Low Fe" to feed *D. pulex*.

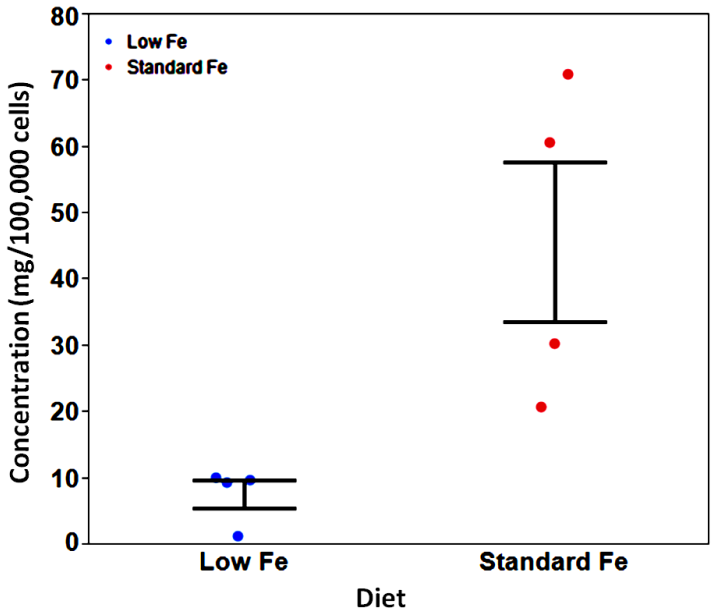

Figure 3. Comparison of Fe Concentration in *A. falcatus* Grown in Standard and Low Fe Media. Error bars represent SEM.

Table 2. Data Summary for Fe concentration in *A. falcatus*

Diet	**N=**	**Mean ± SEM (mg /100,000 cells)**
Low Fe	4	7.65 ± 2.104
Standard Fe	4	45.69 ± 11.971

D. pulex

After the *Daphnia* were fed a standard or low Fe diet, the Fe concentration of the *Daphnia* was measured; the summary of the total Fe levels in *D. pulex* are shown in Table 3. Fe levels of *D. pulex* on the low-Fe diet was significantly lower compared to those who are fed a standard Fe diet (means = 8.40 µg vs. 21.95 µg/*D. pulex; t* = 0.0154) which suggests that the Fe content in *Daphnia* decreases when fed algae that was grown in media with reduced Fe. From this, we have established a low Fe model in *D. pulex,* induced by a low Fe dietary intake in the animals.

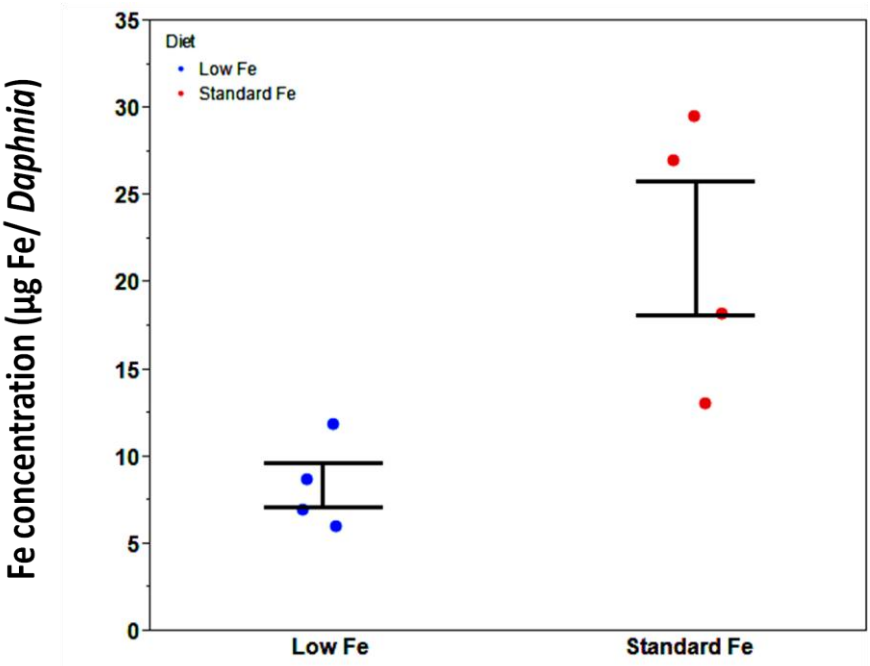

Figure 4. Fe Concentrations of *D. pulex* Fed Standard and Low Fe Diets. Error bars represent SEM

Table 3. Summary of Fe Concentration in *D. pulex*

Diet	**N=**	**Mean ± SEM (µg /*D. pulex*)**
Low Fe	4	8.40 ± 1.29
Standard Fe	4	21.95± 3.83

Lifespan and Reproduction

Lifespan

Daphnia were exposed to MeHg to observe any alterations in lifespan of standard and low Fe diets. Prior to and after the 24hr exposure, *Daphnia* were counted to examine any differences in acute mortality, summarized in Table 4. All *Daphnia* on the standard Fe diet survived the 24-hr exposure while more than 83% survived from those in the low Fe diet group. Overall, there was a significant difference in the acute mortality effect among the treatment groups (Pearson's Chi square = 21.572; $p < .0001$). Particularly, there was a significant effect between the two diets with the paired exposure (MeHg: Pearson's Chi square = 11.083; $p = 0.0009$; Vehicle: Pearson's Chi square = 10.351; $p = 0.0013$); however, the mortality of the vehicle exposed and MeHg exposed *D. pulex* on the same diet were not significantly different (Low Fe: Pearson's Chi square = 0.063; $p = 0.8025$). As a result, it is suggested that low Fe, but not MeHg exposure, increases the acute mortality of *D. pulex* in either the vehicle or MeHg 24 hour exposure compared to the standard Fe group.

Table 4. Acute Mortality after MeHg and Vehicle Exposure

Diet	**Treatment (Dose ng MeHg/L)**	***N* before exposure**	***N* after exposure**	**% Survived**
Low Fe	MeHg (1600)	60	50	83.33
Low Fe	Vehicle (0)	60	51	85.00
Standard Fe	MeHg (1600)	64	64	100.00
Standard Fe	Vehicle (0)	61	61	100.00

From the surviving exposed and not exposed *Daphnia* offspring, 20 samples were haphazardly selected in each group to evaluate differences in lifespan and reproduction. The lifespan data, classified by diet and treatment groups, is shown on Table 5. Since there was no significant difference in lifespan between vehicle and no exposure controls in their paired diets (Standard Fe: Wilcoxon= 0.4865, shown in Figure 5A.; Low Fe: Wilcoxon= 0.7508, Figure 5B.), the two groups were combined as a control to compare with MeHg exposures, summarized in Table 6. With combined controls, *D. pulex* that were fed a low-Fe diet did not have a significant difference in lifespan compared to the standard Fe diet (Wilcoxon= 0.2953, Figure 6A.). Moreover, a significant impact on lifespan was not observed in *Daphnia* that were exposed to MeHg at a concentration of 1600ng/L compared to its paired dietary controls (Standard Fe: Wilcoxon= 0.9374, Figure 6B; Low Fe: Wilcoxon= 0.8411, Figure 6C.), but we did note there was a slight increase in lifespan when exposed to MeHg. Moreover, there was no significant affect on *D. pulex* in low-Fe diets and MeHg exposure when compared to the standard Fe control, shown in Figure 6D. (Wilcoxon = 0.3307). Also, there was no significant effect on lifespan when comparing MeHg exposed in standard Fe versus low Fe diets (Wilcoxon = 0.4757, Figure 6E.). These data suggests that neither MeHg exposure nor standard or low-Fe diets affects lifespan in *D. pulex*.

Table 5. Summary for Lifespan of *D. pulex* by Diet and MeHg Exposure

Diet	Treatment (Dose ng MeHg/L)	N=	Number Censored	Median (Days)	Mean ± SEM (Days)
Low Fe	MeHg (1600)	20	0	48	44.70 ± 2.64
Low Fe	No Exposure (0)	20	0	44	35.40 ± 5.11
Low Fe	Vehicle (0)	19	1	41	31.42 ± 4.71
Standard Fe	MeHg (1600)	19	1	49	45.74 ± 3.56
Standard Fe	No Exposure (0)	20	0	46	41.90 ± 4.83
Standard Fe	Vehicle (0)	18	2	41	36.67 ± 5.51
Combined		**116**	**4**	**45**	**39.29 ± 1.85**

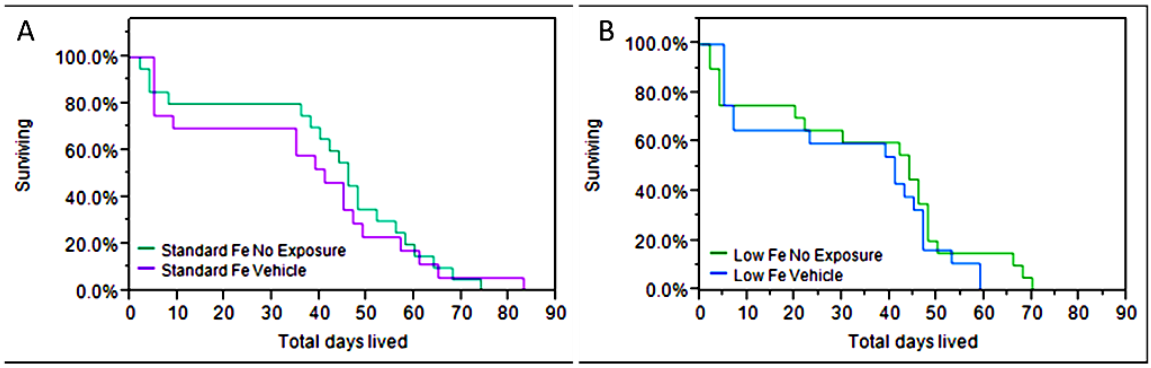

Figure 5. Survival Curves for Comparing Vehicle and No Exposure Controls for (A) Standard Fe and (B) Low Fe.

Table 6. Data Summary of Lifespan of *D. pulex* by Diet and Dose (Controls Combined)

Diet	Treatment (Dose ng MeHg/L)	N=	Number Censored	Median (Days)	Mean ± SEM (Days)
Low Fe	MeHg (1600)	20	0	48	44.70 ± 2.64
Low Fe	Control (0)	39	1	42	33.42 ± 3.46
Standard Fe	MeHg (1600)	19	1	49	45.74 ± 3.56
Standard Fe	Control (0)	38	2	45	39.32 ± 3.62
Combined		**116**	**4**	**45**	**39.29 ± 1.85**

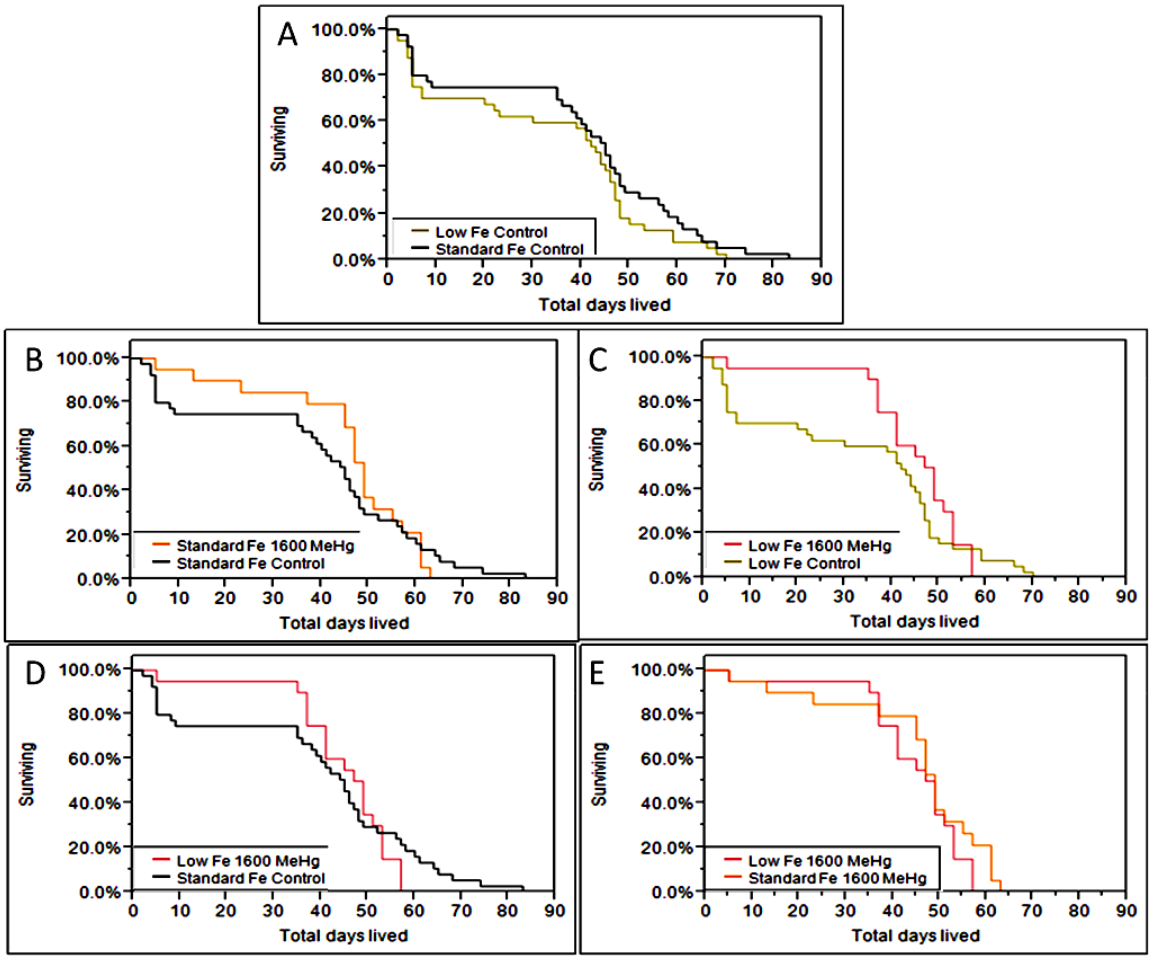

Figure 6. Survival Curve Comparisons for (A) Standard vs. Low Fe controls, (B) Standard Fe control vs. Standard Fe MeHg, (C) Low Fe Control vs. Low Fe MeHg, (D) Standard Fe Control vs. Low Fe MeHg (E) MeHg exposure in Standard Fe and Low Fe

Time to First Reproduction

Summary statistics, including mean and median time to first reproduction, are listed on Table 7. A total of 93 samples reached reproductive age and were able to be analyzed. All surviving *Daphnia* were able to reproduce within 8-14 days. In both standard and low-Fe diets, the vehicle control group were significantly different compared to the no exposure control group (Standard Fe: Wilcoxon = 0.0056; Low Fe: Wilcoxon <0.0001); thus, control groups were not combined for this analysis. Also, Wilcoxon test results among treatment groups for maturity time are listed on Table 8.

The no exposure controls were not significantly different in standard or low Fe diet. Also, compared to the no exposure groups in standard and low Fe diets, those that were exposed to MeHg or vehicle significantly delayed the time to first reproduction; however, MeHg and vehicle control were not significantly different from each other when compared in standard and/or low Fe diets.

Table 7. Summary for Time to First Reproduction

Diet	**Treatment (Dose ng/L)**	**N=**	**Median (Days)**	**Mean ± SEM (Days)**
Low Fe	MeHg (1600)	19	13	12.58 ± 0.246
Low Fe	No Exposure (0)	15	10	10.00 ± 0.338
Low Fe	Vehicle (0)	12	13	12.50 ± 0.261
Standard Fe	MeHg (1600)	19	13	12.58 ± 0.289
Standard Fe	No Exposure (0)	16	10	10.88 ± 0.446
Standard Fe	Vehicle (0)	12	13	12.67 ± 0.333
Combined		**93**	**13**	**11.87 ± 0.169**

Table 8. Wilcoxon Test for Time to First Reproduction in *D. pulex*

Diet		**Standard Fe**		**Low Fe**		
	Dose	**Vehicle**	**1600ng/L**	**No Exposure**	**Vehicle**	**1600ng/L**
Standard Fe	**No Exposure**	0.0056*	0.0014*	0.1966	0.0083*	0.0014*
	Vehicle		0.8148	<0.0001*	0.7436	0.8434
	1600ng/L			<0.0001*	0.9425	0.9453
Low Fe	**No Exposure**				<0.0001*	<0.0001*
	Vehicle					0.8779

* indicates significant differences

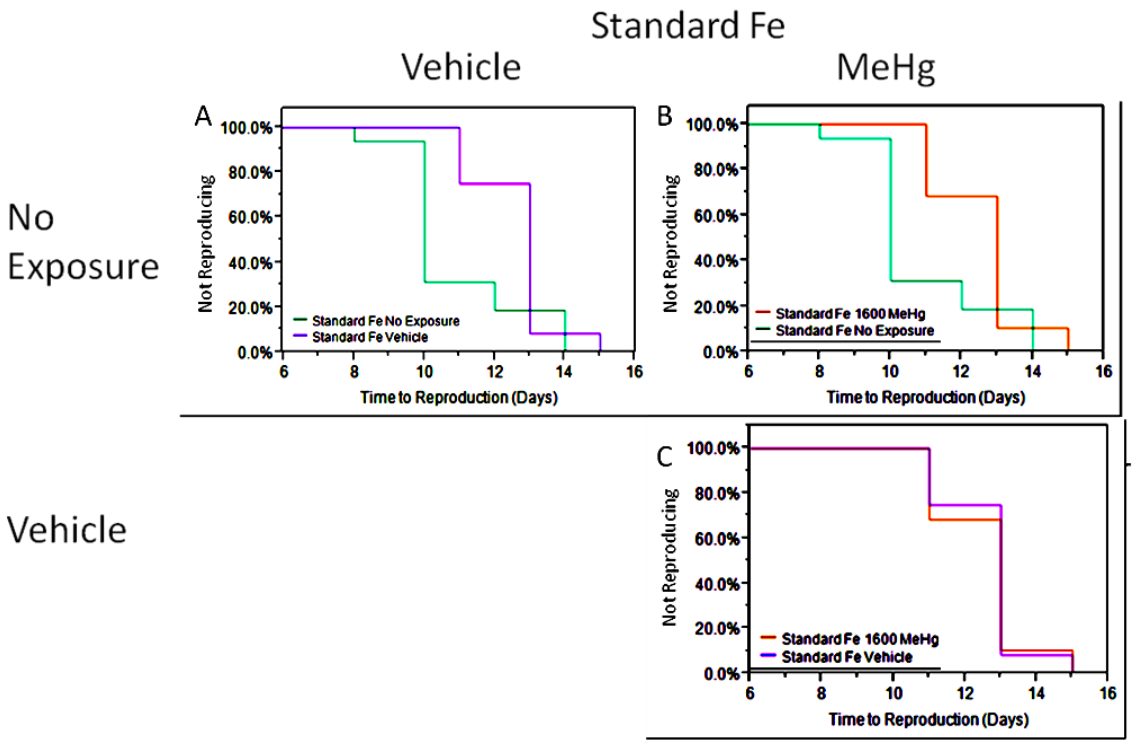

Figure 7. Time to First Reproduction Curves in Standard Fe Diet between (A) No exposure and Vehicle Controls; (B) No exposure control and MeHg; and (C) Vehicle Control and MeHg

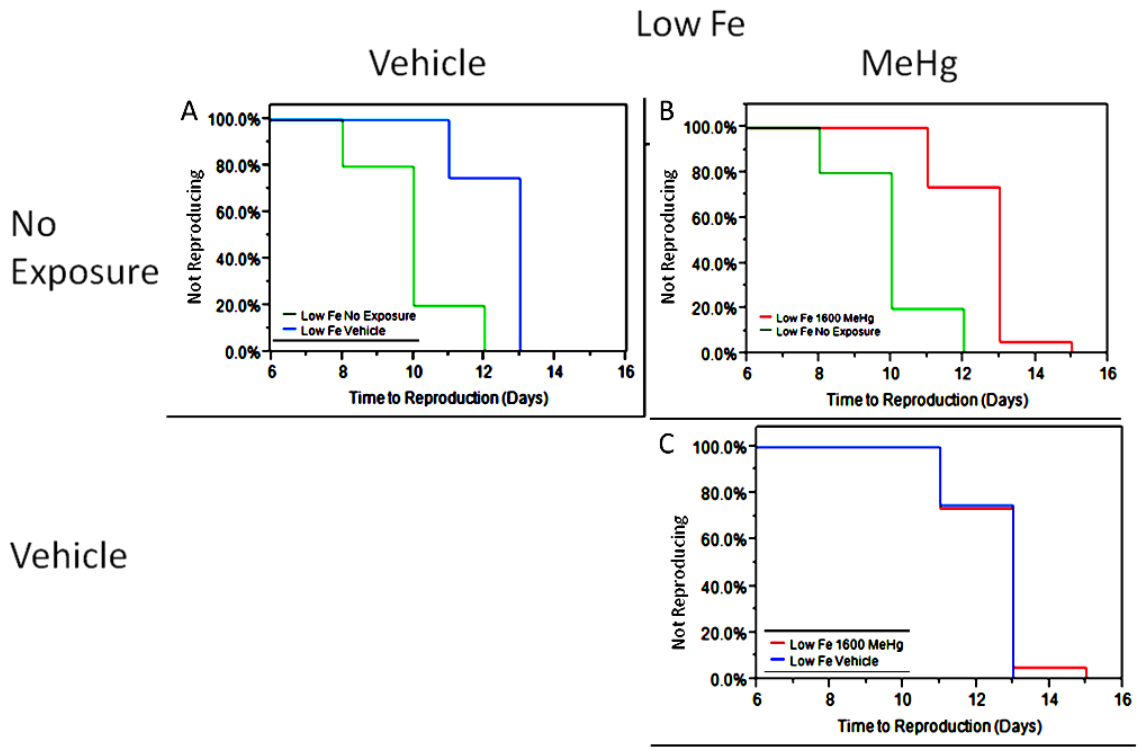

Figure 8. Time to First Reproduction Curves for Low Fe Diet between (A) No exposure and Vehicle Controls; (B) No exposure control and MeHg; and (C) Vehicle Control and MeHg

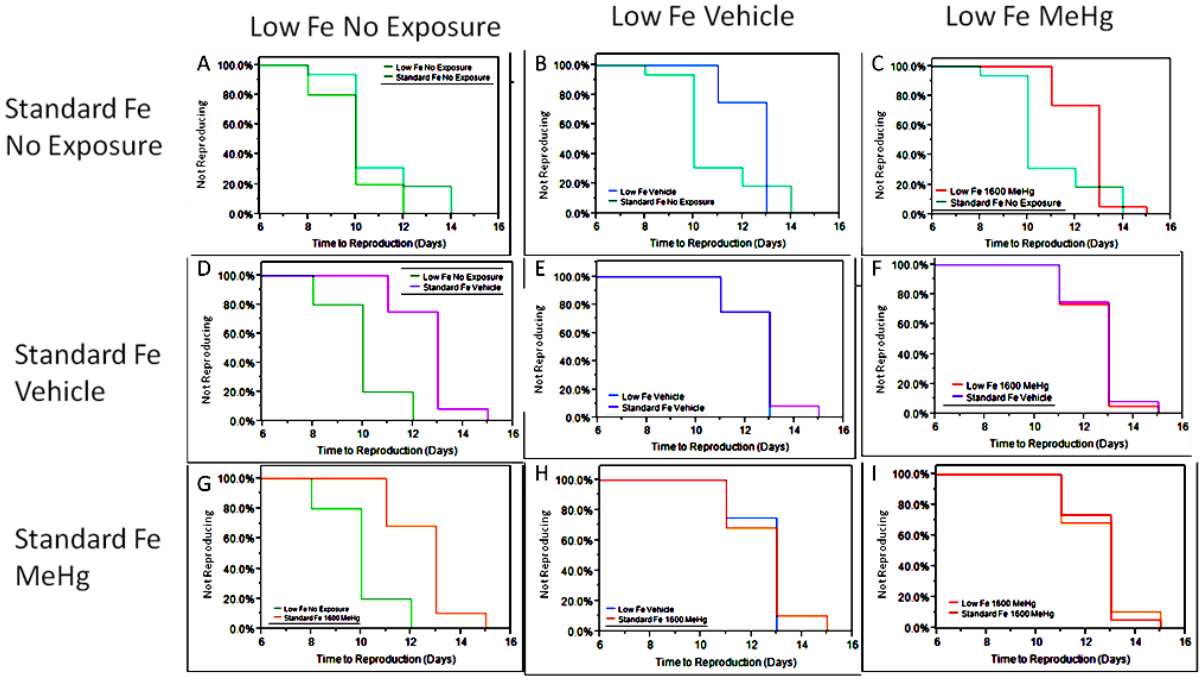

Figure 9. Low Fe vs. Standard Fe Time to First Reproduction between Exposure Groups

Average Brood Size

Out of 120, 93 *Daphnia* reproduced. One outlier reproduced only one clutch and was excluded; thus, a total of 92 *Daphnia* were used in this analysis. Since there was no significant difference between the vehicle and no exposure controls in both diets (Standard Fe: Tukey's = 0.9942; Low Fe: Tukey's = 1.000), the two treatment groups were combined for further analysis. The average brood size means for each treatment, including the combined controls, were approximately 5 offspring per clutch (Table 9). The average brood sizes were not statistically different between control *Daphnia* that were fed a low Fe diet compared to the standard diet (Tukey's = 0.4136). Furthermore, there was no difference between MeHg exposures and controls in its respective diets (Standard Fe: Tukey's = 0.1678; Low Fe: Tukey's = 0.9997). Also, no difference was seen between the low Fe MeHg treatment group when compared to the standard Fe controls (Tukey's = 0.4407). When comparing average brood size of MeHg exposed *Daphnia* between the two diets, there was no significant difference (Tukey's =0.9455). From this, one can conclude that neither diet nor MeHg exposure changes the average brood size in *D. pulex.*

Table 9. Summary Data of Average Brood Size by Diet and Exposure

Diet	**Treatment (Dose ng/L)**	**N=**	**Mean ± SEM**
Low Fe	MeHg (1600)	19	5.32 ± 0.225
Low Fe	Control (0)	27	5.29 ± 0.188
Standard Fe	MeHg (1600)	18	5.50 ± 0.231
Standard Fe	Control (0)	28	4.88 ± 0.185
Combined		**92**	

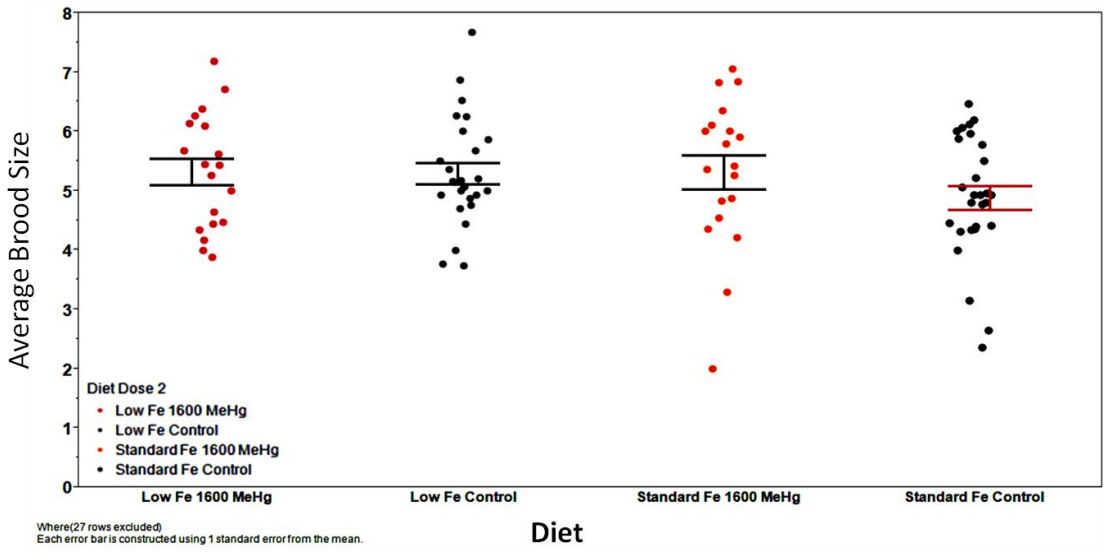

Figure 10. Average Brood Size among Diets and MeHg Exposure. Error bars represent SEM

Reproduction Rate

From the 93 reproducing *Daphnia*, the same one clutch outlier was excluded, leaving a total of 92 for analysis. Since the vehicle and no exposure controls did not significantly differ in each diet (Standard Fe: Tukey's = 0.9889; Low Fe: Tukey's = 0.9991), the control groups were combined for further analysis. With the combined controls, the mean reproduction rate for each treatment group ranges between 1-2 *Daphnia* each day (Table 10.). There was no significant difference between low and standard Fe controls (Tukey's = 0.9985). Also, there was no significant difference in MeHg exposures when compared to its diet paired controls (Standard Fe: Tukey's = 0.8593; Low Fe: Tukey's = 0.9602). Also, there was no significant effect in reproduction rate between the standard Fe controls and MeHg in the low Fe diet group (Tukey's = 0.9179). When *D. pulex* are exposed to MeHg, there was no significant difference between the different diets (Tukey's = 0.5622). Overall, the results suggest that reproduction rate in *D. pulex* is not affected of by differing diets or early-life MeHg exposure.

Table 10. Summary Statistics of Reproduction Rate

Diet	**Treatment (Dose ng/L)**	**N=**	**Mean ± SEM (offspring/day)**
Low Fe	MeHg (1600)	19	1.57 ± 0.102
Low Fe	Control (0)	27	1.63 ± 0.085
Standard Fe	MeHg (1600)	18	1.76 ± 0.104
Standard Fe	Control (0)	28	1.65 ± 0.084
Combined		**92**	

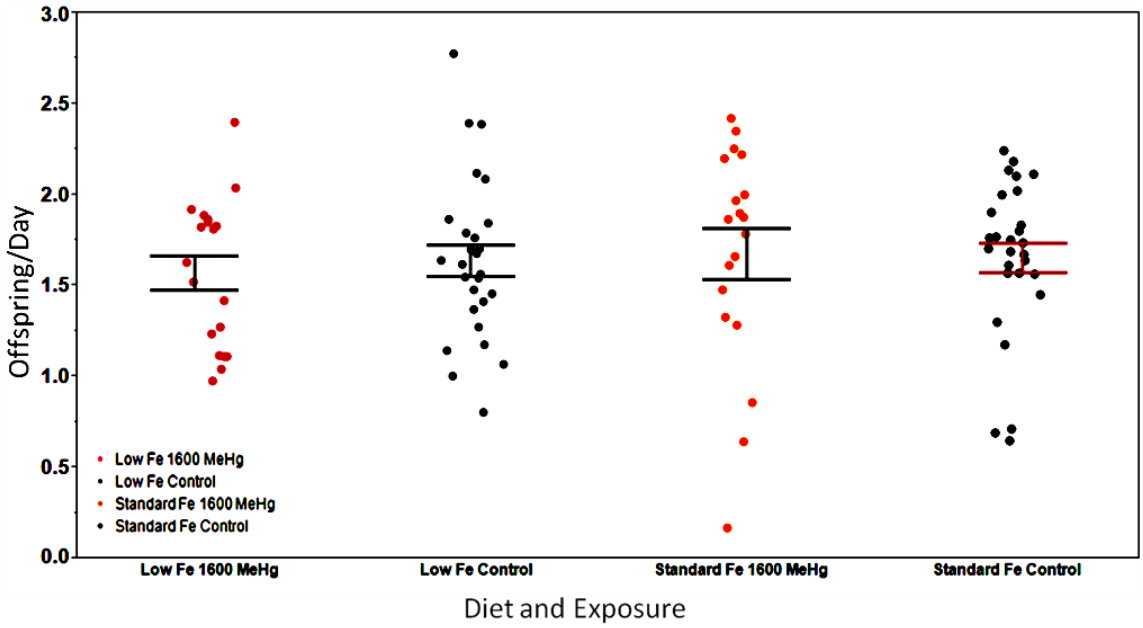

Figure 11. Reproduction Rate among Diets and MeHg Exposure. Error bars represent SEM

Lipid Reserves

Image analysis

A total of 149 images of individual *Daphnia* were analyzed using ImageJ; however, 8 were excluded from statistical analysis due to organisms being crushed during microscopy or the image was out of focus. Thus, this leaves a total of 141 images for statistical analysis, summarized in Table 11.

Table 11. Data Summary for %Lipid based on Oil Red O staining

Diet	Treatment (Dose ng/L)	N=	Range (% Lipid)	Mean ± SEM (% Lipid)
Low Fe	MeHg (1600)	12	(1.47, 8.09)	4.56 ± 0.620
Low Fe	No Exposure (0)	26	(0.037, 5.75)	1.87 ± 0.283
Low Fe	Vehicle (0)	21	(0.001, 3.36)	0.978 ± 0.216
Standard Fe	MeHg (1600)	20	(1.52, 6.21)	4.02 ± 0.322
Standard Fe	No Exposure (0)	42	(0.098, 7.81)	2.89 ± 0.325
Standard Fe	Vehicle (0)	20	(0.029, 5.88)	2.21 ± 0.349

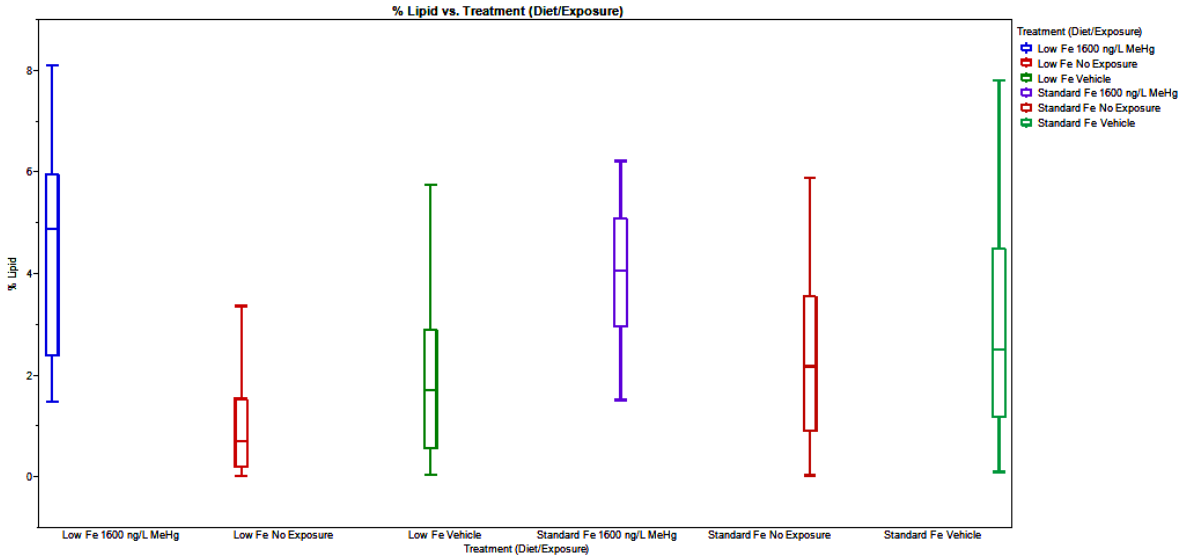

Figure 12. Box plot of %Lipid among Diets and Exposure. Error bars represent the maximum and minimum values. Boxes represent the 25th and 75th percentiles.

The low Fe no exposure group had the lowest mean % lipid (0.978% lipid) while *Daphnia* fed the low Fe diet and exposed to MeHg early in life had the highest mean lipid percentage (4.56% lipid). There is a notable trend between the different exposures, with lipid percentages increased in MeHg exposed compared to controls in each diet. Since increased body size is associated with a heightened % lipids in *Daphnia* illustrated in Figure 13., we decided to use a multivariate linear regression analyses, using the covariates: total body area, amount of *Daphnia* in jars (Jars N), diet, exposed to MeHg, and exposed to MeOH; the model also included the interaction between diet and MeOH

or MeHg. Since the MeHg was dissolved in the vehicle (MeOH), *Daphnia* that was exposed to MeHg was also categorized under the MeOH variable. The model, summarized in Table 12, suggests that MeOH, and the interaction between diet and MeHg showed a significant effect on the lipid/body percentage in *D. pulex*; thus, implying that *D. pulex* that were exposed to MeOH, MeHg or were fed a standard Fe diet, had a higher lipid/body percentage compared to those that did not receive the stated treatments. Also, the model demonstrates that when *Daphnia* were fed a low Fe diet but did not receive MeHg treatment, there was a significant decrease in % lipids.

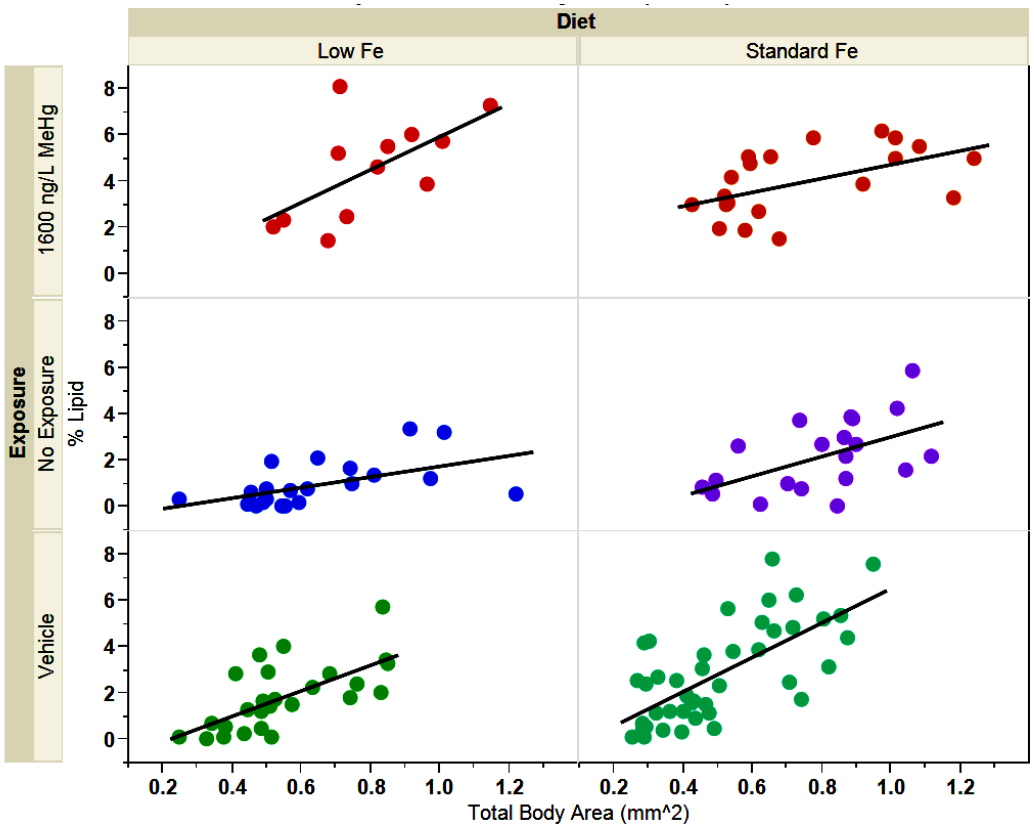

Figure 13. Relationship between %Lipid and Total Body Area by Diet and Exposure

Table 12. Model for % Lipids and its covariates

Covariate	DF	Estimate	SS	F Ratio	Prob>F
Diet (Low Fe)	1	-0.0506	0.1758	0.0916	0.7627
MeOH (No MeOH)	1	-0.8809	53.773	28.004	<.0001*
MeHg (No MeHg)	1	-0.3224	3.266	1.701	0.1944
Jars N	1	0.0018	0.0538	0.0280	0.8674
Total Body Area	1	4.7840	99.922	52.038	<.0001*
Diet*MeHg (Low Fe * No MeHg)	1	-0.3840	12.032	6.266	0.0135*
Diet*MeOH (Low Fe * No MeOH)	1	0.1825	3.135	1.633	0.2036

TAG assay

Results from the image analysis imply *Daphnia* that were exposed to MeHg and fed a standard Fe diet increase lipids compared to the controls. To verify this finding, TAG content was measured via chemical analysis. A total of 15 pooled *Daphnia* samples were included in the analysis. Means of TAG concentration are listed on Table 13. Compared to the image analysis results, the mean TAG concentration showed a similar increase of lipids when *Daphnia* were exposed to MeHg. The sample sizes are small and vary between treatments; therefore, the generalized linear model is used for this analysis. Since the TAG concentrations in no exposure and vehicle controls of each corresponding diets are not significantly different (Standard Fe: Tukey's= 0.2896, Low Fe: Tukey's= 0.6597), the two groups are combined for further analysis to test the effects of TAG content in MeHg exposed groups. With the combined controls, there was no significant difference between MeHg exposure and controls in their respective diets (Tukey's = 0.6811 and 0.9703 for Standard and Low Fe diets, respectively). In addition, MeHg exposed *Daphnia* of a low Fe diet were not significantly different when compared to the standard Fe controls (Tukey's = 0.7684). Also, standard and low-Fe diets did not

significantly alter the TAG content when exposed to either MeHg (Tukey's = 1.000) or not (Tukey's = 0.9175).

Table 13. Data Summary of Triacylglycerol (TAG) Assay

Diet	**Treatment (Dose ng/L)**	**N=**	**Mean ± SE (µg TAG/ animal)**
Low Fe	MeHg (1600)	2	9.92 ± 3.236
Low Fe	Control (0)	4	8.18 ± 2.288
Standard Fe	MeHg (1600)	3	9.94 ± 2.642
Standard Fe	Control (0)	6	6.29 ± 1.868
Combined		**13**	

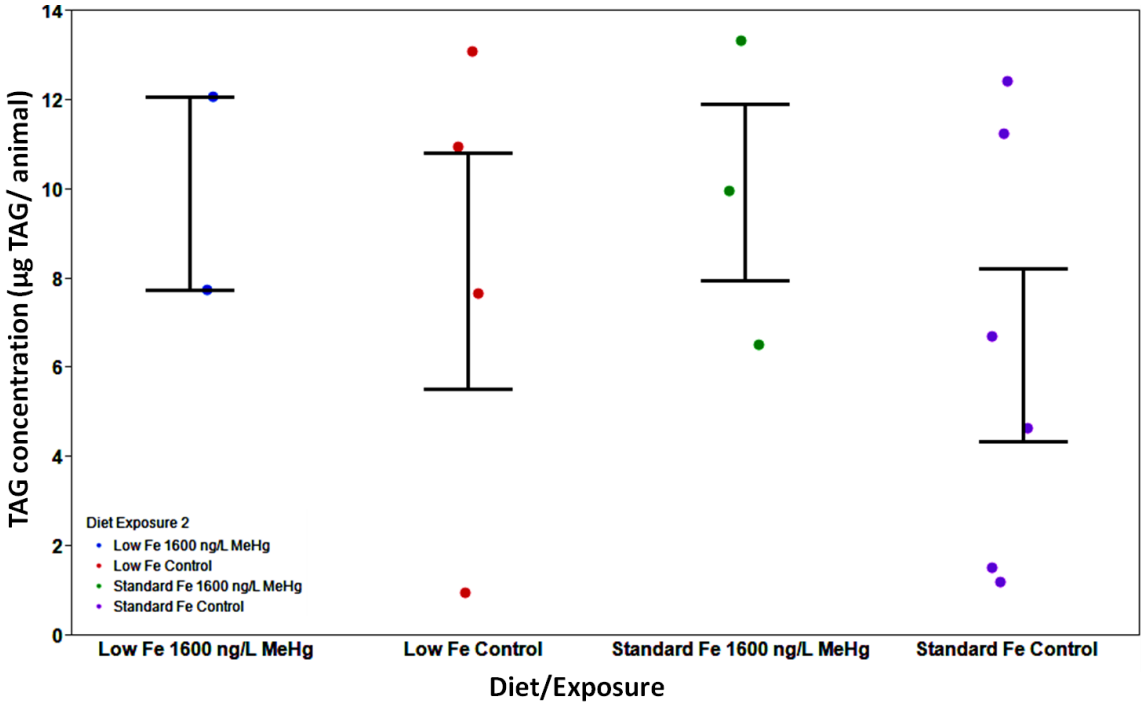

Figure 14. TAG Assay Comparison in Diet and Exposure. Error bars represent SEM.

DISCUSSION

This is the first study that uses *Daphnia* to investigate the interaction between low-level, early-life MeHg exposure and a low-Fe diet. In addition, this study is the first to demonstrate the use of ORO followed by image analysis to stain and quantify storage lipids in *Daphnia*. The purpose of this study was to determine if there is a difference in MeHg toxicity when *Daphnia* were fed a low-Fe diet compared to a standard-Fe diet. From previous studies, we hypothesized that early-life, low-level exposure to MeHg would shorten *Daphnia*'s lifespan and adversely affect its reproduction, as well as decrease its lipid storage; we also believed *Daphnia* on a low-Fe diet would exacerbate the toxicity of MeHg on the basis of a shorter lifespan and reduced reproduction. However, the data from this study did not support our hypotheses. In this current study, we have shown that Fe concentrations were lowered in *A. falcatus* when cultured in low Fe growth media compared to standard Fe growth media. Also, Fe levels in *D. pulex* were reduced when consuming a low-Fe diet. Most importantly, this study did not show any adverse effects to early-life MeHg exposure in terms of lifespan, average brood size, and reproduction rate in *D. pulex*. However, a delay in the start of reproduction is evident when exposed to MeHg or the vehicle in either standard or low Fe diets, suggesting there was a significant stress caused by the additional handling in the exposure paradigm. Also, from the lipid staining analysis, an early-life exposure to MeHg under a low Fe diet increases lipid storage in *D. pulex* while a low Fe diet alone accounts for an insignificant

decrease in lipid storage. Quantification of TAG via chemical assays did not show any significant differences in *Daphnia* that were either fed a low-Fe diet or were exposed to MeHg.

Iron Deficiency

Fe is essential for *Daphnia* in hemoglobin synthesis and reproduction (Engle 1985; Smaridge 1956; Dave 1984). However, based on the present study, there are no significant differences in lifespan or reproduction in *D. pulex* fed a low-Fe diet. The current study's low Fe effects on lifespan are not consistent with past studies indicating that ID or low Fe levels increase the risk of mortality in humans and rodent models (Gambling et al. 2003; Kilp et al. 2013; Dave 1984), but is consistent with another study (Tielsch et al. 2006) indicating Fe has no significant effect on childhood mortality. Concerning reproduction, data from the present study is consistent with past studies that have observed no significant effects (Gambling et al. 2002; Gambling 2003).

Using an enzymatic assay to measure TAG in *Daphnia* suggested there was no significant difference in lipid content between the differing diets. This finding was not consistent with past studies, indicating that ID increases lipid content (Nandyala et al. 2013; Stangl & Kirchgessner 1998; Davis et al. 2012). However, the mean lipid concentration, listed on Table 13, between the controls of both low and standard Fe groups imply there may be an increase of lipids in *Daphnia* fed a low-Fe diet, but did not reach statistical significance due to high variation in different runs of the assay and small sample size. Therefore, additional assays for TAG concentrations with a larger sample size may be necessary. Interestingly, from ORO staining followed by image analysis,

low-Fe diet in *D. pulex* was not a sole factor for the alteration in lipid content, but the interaction between diet and exposure to MeHg was responsible for the increase of lipids.

To our knowledge, this is the first study that investigated the effects of low-Fe diets in *D. pulex*. Past *Daphnia* studies addressed the effects of various Fe concentrations in media (Dave 1984; Bošnir et al. 2013), but neither addressed outcomes relating to Fe in the diet. Accounting for dietary Fe may clarify the reason for variability in toxic concentrations of Fe in the media.

Methylmercury

The experimental concentration of MeHg is higher than most recorded concentrations in surface water bodies (> 5ng/L) (ASTDR 1999); for example, a study that measured Canadian surface water levels reported between 0.02 and 4 ng/L while a study in California wetlands reported MeHg between 0.1 and 37 ng/L (Hall, Baron, & Somers 2009; Alpers et al. 2013). In addition, the experimental MeHg concentration is higher compared to the EC_{50} that may affect *D. pulex*'s reproduction from chronic exposure (30 days) to MeHg (707 ng MeHg/L); however, it is lower than the LC_{50} of the Daphnia for 24-hr exposure (31205 ng MeHg/L) as well as for the 48-hour period (5700 ng MeHg/L) (Tiang-yi & McNaught 1992). At low levels, MeHg did not affect the lifespan in *D. pulex,* similar to past studies in *C. elegans* and other organisms (Helmcke et al. 2009; Schroeder & Mitchener 1974; Hammerschmidt et al. 2002). Furthermore, there was no significant difference in reproduction, unlike previous studies that suggested that exposure to MeHg adversely affected reproduction (Albers et al. 2007; VanDuyn et al. 2010). Interestingly, a significant delay in time to first reproduction was not observed

in *D. pulex*; contrasting to the delayed spawning that was observed in MeHg exposed fathead minnows (Hammerschmidt 2002).

TAG levels were slightly, but not significantly increased in *Daphnia* exposed to MeHg. This finding is in contrast to past studies that suggested MeHg reduced TAG concentrations in rodents (Kawakami 2011; Sood et al. 1997; Shirai et al. 2007). Results from the ORO staining and analysis suggests that exposure to MeHg increases lipid storage, which is not consistent to past studies in Northern Pike and mice (Drevnick et al. 2008; Kawakami et al. 2011).

TAG Assay

The quantification of triacylglycerol (TAG) using an enzymatic assay kit did not show a correlation to the ORO image analysis in terms of %lipid content (data not shown). This finding differed from past studies, stating that lipids stained with ORO did correspond to the amount of lipids quantified with biochemical assays (Ramírez-Zacarías, Castro-Muñozledo, & Kuri-Harcuch 1992; Ge et al. 2010). Based on the present analysis, using commercial kits for quantifying lipids in *D. pulex* is not optimal for the following reasons: 1.) Due to pooled samples, body size was not accounted for in the TAG assay; this study has shown that % lipid is correlated with the body size of *D. pulex*, which is synonymous with Tessier and Goulden's (1982) observation of lipids in *Daphnia*. 2.) Individual *Daphnia* age varied within 2 days; thus, *Daphnia* were at varying life stages and sizes, which could impact the lipid concentration in *Daphnia*; and 3.) high glycerol content was observed in the wells.

Limitations

There are several limitations in this study. For this experiment, *Daphnia* underwent an acute (24hr) exposure to MeHg to investigate any adverse long-term effects; however, chronic exposure to MeHg would be more consistent with humans as well as other studies. A previous study estimated that half of the *Daphnia* population reduces reproduction when chronically exposed to 707 ng/L of MeHg for 30 days (Tian-yi & McNaught 1992). In this study, there were no differences in the reproduction rate when *Daphnia* were subjected to an acute 24 hr. exposure to a higher concentration of 1600 ng/L MeHgCl.

Due to the presence of selenium in the media in which is necessary for *D. pulex* growth during the 24 hr MeHg exposure, the effects of MeHg may have been countered during the exposure period (Goyer 1997). The protective effects of selenium against MeHg have also been shown in previous studies (Bjerregaard & Christensen 2012). Also, since the animals were checked every other day in the present study, the maturation time could be further characterized using a shorter time between observations in future studies.

During ORO staining, the fusion of adjacent lipid droplets may occur with the presence of alcoholic solvents during the staining process or fixing the samples; thus making the individual lipid droplets appear larger, although total percent lipid should not be affected (Mehlem et al. 2013). Also, conducting the image analysis of lipids with software is time-consuming, especially with larger data sets; thus, optimizing time-saving methods for ORO in *D. pulex* is recommended.

Another limitation to this study is that the lipid content of the organism may also differ since *Daphnia* is affected by its food quality (Ahlgren et al. 1990) and not solely

due to ID. Furthermore, the lipid content of the algae may differ, with algae grown in Fe-depleted environments, although we would expect this to cause a slight increase in TAG content (Fan et al. 2014).

Future Work

Additional research on the outcomes of various dietary Fe concentrations in *Daphnia* needs to be investigated. Also, more studies could be conducted to find the relationship between early-life MeHg exposure and low Fe in *Daphnia* without selenium during exposure or throughout lifespan. A standardization of carbon and lipid content in the diet would be ideal. Future work would also include optimizing lipid quantification in *D. pulex*. Various methods of lipid quantification in *D. pulex* include adipose conversion through the extraction of ORO from stained lipids to spectrophotometrically quantify the presence of fat, along with use of a standard curve (Ramírez-Zacarías, Castro-Muñozledo, & Kuri-Harcuch 1992), as well as the use of the micromethod to quantify small aquatic invertebrate samples, described by Gardner et al. (1985).

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