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The effect of a low iron diet and early life methylmercury exposure in *Daphnia pulex*

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Abstract

Iron (Fe) deficiency increases risk for adverse health outcomes in humans; however little is known about the potential interaction with methylmercury (MeHg) exposure. Studies testing multiple stressor hypotheses are expensive and time consuming in mammalian model systems; therefore, determining relevance of alternative models is important. *Daphnia pulex* were fed standard or low-Fe diets of freshwater algae, *Ankistrodesmus falcatus*. MeHgCl (1600 ng/L) or vehicle was added to culture media for 24 h during early life, and the combinatorial effects of a low-Fe diet and MeHg exposure on lifespan, maturation time, and reproduction were evaluated. Lipid storage effects were measured using image analysis of Oil Red O staining and triacylglyceride quantification. Our results show a dose-dependent reduction in lifespan in *D. pulex* fed low Fe diets. Lipid analysis suggests an interactive effect of diet and MeHg exposure, with MeHg exposure increasing lipid storage in *D. pulex* fed a low-Fe diet. These findings suggest the effects of dietary iron intake and early life MeHg exposure in *D. pulex* may be mediated by changes in energetics that result in differential lipid storage. Therefore, lipid storage in *D. pulex* may be a useful screen for detecting long-term effects of multiple stressors early in life.

Keywords

Metals; Iron deficiency; Methylmercury; Lipid storage; *Daphnia*

1. Introduction

Iron deficiency (ID) is the most common micronutrient deficiency in the world and is prevalent among infants, young children, and women at reproductive age (CDC, 2002).

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Transparency document

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.fct.2016.01.012>.

Prenatal ID is associated with reduced conception rate, perinatal and maternal infection and mortality, increased blood pressure, increased serum lipid profiles, and reduced myelination and subsequent neurocognitive effects in humans and rodent models (Zimmermann and Hurrell, 2007; Brabin et al., 2013; Gambling et al., 2003; Li et al., 2014; Sherman, 1979; Yu et al., 1986; Radlowski and Johnson, 2013).

MeHg is a known developmental neurotoxicant, with prenatal exposure resulting in neurocognitive impairments (Karagas et al., 2012). Some evidence suggests that dietary deficiencies may influence the toxicity of MeHg; although due to the collinearity of nutritional factors in fish eating populations, it is unclear whether specific micronutrient deficiencies directly affect MeHg toxicity (Davidson et al., 2008). Fonseca et al. (2014) found a weak association between hair mercury levels and hemoglobin, but not ferritin blood levels in Amazonian women.

Both MeHg exposure and ID have been shown to affect lipid homeostasis and MeHg exposure is associated with increased risk of myocardial infarction (Stern, 2005). MeHg induced hypercholesterolemia in mice leads to cerebellar glial activation, suggesting MeHg induced dyslipidemia plays a role in both the cardio and neurotoxic effects of MeHg (Moreira et al., 2012). With regard to ID, triglyceride (TAG) serum levels were significantly elevated among Indian adults with ID compared to those who were not; subsequently, Fe therapy among the ID participants significantly decreased TAG levels after 3 months (Nandyala et al., 2013). In rodent studies, liver TAG levels of ID rats were not affected while serum TAG levels were heightened compared to Fe sufficient rats (Stangl and Kirchgessner, 1998).

Although rodent models of ID exist (Rao and Jagadeesan, 1995), little is known regarding interactions with other stressor exposures early in life. Mechanisms of iron homeostasis are highly conserved (Askwith and Kaplan, 1998), suggesting non-vertebrate organisms may offer an efficient and cost-effective method for screening multi-stressor hypotheses. Indeed, numerous research groups, and federal and international agencies are actively developing alternative models to reduce, refine, and replace mammalian species in toxicological testing of environmental chemicals, foods, and drugs (NRC, 2007; Rovida et al., 2015). Our group is currently evaluating the utility of the freshwater crustacean, *Daphnia pulex*, as a model for screening multi-stressor exposures across the lifespan. In this study, we evaluate early-life MeHg exposure and ID interactions in *D. pulex*.

D. pulex is already widely used for ecotoxicological studies (Shaw et al., 2007; LeBlanc, 1980; Lampert, 2006) and is an emerging biomedical research model organism (NIH, 2013) with advantageous traits including parthenogenic (clonal) reproduction, a short lifespan (60 days), well-characterized ecology and responses to environmental stimuli, release of live offspring, transparency throughout adulthood, and recently developed genomics tools (NIH, 2013; Lampert, 2006; Shaw et al., 2008; Colbourne et al., 2011). In this study, we take advantage of *Daphnia's* transparency through adulthood, developing a methodology for examining lipid storage through whole animal image analysis.

Lipid storage, mostly composed of TAG, and reproductive output of *D. pulex* are highly dependent on food quantity and quality (Tessier and Goulden, 1982; Goulden and Place, 1990; Kilham et al., 1997). Arzate-Cárdenas and Martínez-Jerónimo (2012) suggested that energy reserves in *Daphnia* are reliable stressor biomarkers to evaluate the effects of hexavalent chromium toxicity at sublethal doses. In addition, DeCoen and Janssen (2002) concluded that lipid reserves in *Daphnia* are considered a sensitive biomarker of population level outcomes after exposures to chemical stressors.

Past studies have evaluated Fe and its toxicity in *Daphnia* (Dave, 1984; Bošnjir et al., 2013); however, to our knowledge, no study investigated reduced dietary Fe levels and its effects in *Daphnia*. In addition, several studies have investigated mercury's toxicity in *Daphnia*. For example, age-specific birth rate and total offspring of *D. pulex* was reduced when exposed between 10 ng/L and 1000 ng/L MeHg every 3 days throughout lifespan; a gradual increase in early deaths was also observed (Tian-yi and McNaught, 1992). Doke et al. (2014) found a significant decrease in lifespan after a 24 h early life exposure to 1600 ng/L MeHg, in *D. pulex*.

The purpose of this study is to investigate the effects of a low Fe diet and the effect of an early-life, acute exposure to MeHg in *D. pulex* on lifespan, reproduction and lipid storage. We hypothesized when *D. pulex* are fed a low-Fe diet, the toxicity associated with early exposure to MeHg will become exacerbated.

2. Materials and methods

2.1. Model organism

The *D. pulex* culture was established from clones sent from Dr. Joe Shaw's laboratory at Indiana University, where the clonal line had been in culture for at least 8 years and has high hybridization efficiency to the *D. pulex* TCO microarray (Shaw et al., 2007; Asselman et al., 2013). Clones have been maintained in our laboratory since 2011, and we have previously evaluated dose-response effects of MeHg on lifespan in this clonal line (Doke et al., 2014). *Daphnia* were maintained in COMBO media as described by Kilham et al. (1998) at a density of 20 animals per 1 L beaker. COMBO is reconstituted water that has been shown to support *Daphnia* growth and reproduction (Kilham et al., 1998). *Daphnia* were kept in a temperature-controlled chamber ($22.5\text{ }^{\circ}\text{C} \pm 0.5\text{ }^{\circ}\text{C}$, Percival, I36NLXC8) on a 12:12 light:dark cycle. Similar to previous research (Shaw et al., 2007; Doke et al., 2014), cultures were fed freshwater phytoplankton, *Ankistrodesmus falcatus*, every 48 h, at a concentration of 80,000 cells/mL, unless as explained below. Our culture protocol is designed to ensure clonal reproduction is maintained with regular media changes and careful control of temperature, light and feed rate. Resting eggs are found on rare occasion and are immediately removed. Resting eggs take several weeks to months of exposure to cold and dark prior to hatching (Schwartz and Hebert, 1987), therefore we are confident the clonal make-up of our culture has been maintained, although genetic drift may lead to differences over time (Spitze, 1993).

2.2. Standard and reduced iron algae culture

A. 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 (Kilham et al., 1998; Doke et al., 2014). To reduce the Fe content in algae, addition of the micronutrient FeCl_3 stock solution (3.15 g/L of Fe (III) chloride hexahydrate, Fisher Scientific, Product #S25317A) was reduced to create media with 50% (1.58 mg/L), 10% (0.32 mg/L), or no Fe compared to the standard growth medium concentration (3.15 mg/L). The addition of the Fe chelating agent, EDTA, was also reduced by the same percentages. Algal cells were harvested via centrifugation and/or filtration and washed with COMBO 3 times.

To determine the feeding rate, a standard curve was developed relating cell number estimated by counts using a hemocytometer to optical density readings at 680 nm wavelength on a Beckman Coulter, DU® 800 spectrophotometer (Doke et al., 2014). 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 and likely nutrition received by *D. pulex* (Kosakowska et al., 2004; Fan et al., 2014; Weers and Gulati, 1997).

2.3. Iron quantification

Iron content was determined in *A. falcatus* grown in media containing no added Fe, 10%, 50% and standard Fe media ($N = 3$ for each diet) and in *Daphnia* fed with *A. falcatus* grown in either standard or 50% reduced Fe media ($N = 4$ for each diet). *Daphnia* of various ages (14–51 days) went through a 24 h fasting period before harvesting to determine Fe levels to minimize the effect of algae in gut.

Fe was quantified using an acid-based commercial assay (QuantiChrom™ Iron Assay Kit BioAssay Systems DIFE-250, Hayward CA). Both algal and *Daphnia* samples were sonicated (Branson Sonifier 250) with 10 bursts at a pressure of 30 psi. The sonicated mixture was incubated with reaction mix at room temperature for 40 min and then centrifuged at max speed (13,000 RPM/g) for 10 min. 50 μL –200 μL were loaded into wells of a clear bottom plate, filling with deionized Ultrapure (PURELAB, Elga LabWater) water to reach 250 μL . Standards and samples were read by a colorimetric plate reader (Spectramax Plus 384) at peak absorbance of 590 nm. The final concentrations for algae and *Daphnia* were expressed in mg Fe/100,000 cells and μg Fe/mg *Daphnia* tissue or μg Fe/number of *Daphnia*, adjusting for cell count and mass or number of animals, respectively (García-Casal et al., 2007).

2.4. Early-life methylmercury exposure

For the stock solution, 10 mg of methylmercury II chloride (CH_3HgCl analytical standard, Sigma Aldrich Prod. # 33368) was dissolved in 1.7 mL of methanol and then added to 1 L deionized Ultrapure water. *D. pulex* neonates (2 days) from either the standard or 50% Fe diet were exposed to media with a concentration of 1600 ng/L CH_3HgCl for 24 h without food. This dose level was based on our previous dose finding study, showing this dose affected lifespan and reproduction (Doke et al., 2014). To verify the effects of MeHg

exposure alone, and to account for the potential effects of a 24 h period without food and the increased handling required in the treatment protocol, each treatment group was paired with a no exposure control and a vehicle control (0.2 μ L/L MeOH).

2.5. Lifespan & reproduction

To examine the effect of ID, offspring (ages 0–2 days) were fed either standard, 50%, 10% or No Fe diet continuously until death. Age at first reproduction, total reproduction at 15, 23, and 25 days of age, and lifespan were recorded for at least 3 generations.

To examine the combinatorial effect of a low (50%) Fe diet and early life MeHg exposure, *Daphnia* were acclimated to either low (50%) or standard Fe diets for at least two generations. The parents of experimental animals were kept in 1 L beakers or individually kept in 50 mL polypropylene centrifuge tubes and checked every 48 h for offspring. All offspring after the third clutch were used for experiments. The day the *Daphnia* are harvested from the parental jar is labeled as “Day 0”. Following the 24 h exposure to MeHg or vehicle (Day 1), at least 20 *D. pulex* were individually placed in 50 mL centrifuge tubes with 30–50 mL COMBO media, and fed with either standard or low (50%) 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 h. Age at first reproduction, average brood size, total reproduction and lifespan was calculated for each *Daphnia*. Average brood size is calculated by dividing the total number of offspring over the total reproduction cycles while lifespan reproduction is calculated as the total offspring divided by total days lived.

2.6. Oil Red O (ORO) staining

ORO is a lipid soluble dye that specifically stains neutral lipids and cholesterol esters (Ramírez-Zacarias et al., 1992). Modeling from Yen et al. (2010) and Soukas et al. (2009), a stock solution was prepared by adding 0.5 g of ORO powder (Sigma Aldrich) to 100 mL of 2-propanol alcohol and allowed to equilibrate at room temperature for several days. Within 15 min prior to staining, the stock solution was diluted with 40% of Ultrapure water and set at room temperature for 10 min and then was filtered using a 100 μ m nylon mesh strainer. 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 h.

Live, 6–8 day old *D. pulex* were placed in microcentrifuge tubes and water was removed. 1 mL 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 45–60 min. The stained specimens were then washed with 70% ethanol twice and were fixed using 70% ethanol on a concave microscope slide. Fixed *D. pulex* were placed under a microscope (Olympus BX41) at 4 $\times$ magnification. 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.

Analysis of the ORO stained *Daphnia* was conducted using ImageJ freeware (Rasband, 2014). To visualize the droplets easily, the dark background was subtracted. The thresholding method was set on default while the threshold color was set on red and 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, the images were adjusted according to color threshold, highlighting only the red lipid signal (Deutsch et al., 2014). The image was then converted to a grayscale image using the red, green, and blue stack. The green channel was selected and thresholded. To measure lipid area percentage (Stamps and Linit, 1995), *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.

2.7. 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 1.5 mL 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 and 100 °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 10–50 μ L of supernatant was placed in a black 384 well plate in 10 μ L intervals for two columns; in wells containing less than 50 μ L of sample, the TAG buffer filled the wells to reach 50 μ L. To measure glycerol background, 2 μ L of TAG buffer only was added to one column of samples; whereas, 2 μ L of lipase was added to the other column and the standard column. The plate was incubated at room temperature for 20 min to convert TAG to glycerol and fatty acids. After incubation, 50 μ L 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 min. The prepared plate was analyzed in a fluorometric plate reader (Spectramax Gemini XPS) at excitation/emission phase of 535/590 nm. Standard curve and dilutions were analyzed, with the glycerol backgrounds subtracted for the samples, and were adjusted by weight. TAG concentrations were measured across dilutions for each biological replicate ($N = 15$ total samples, with at least 2 biological replicates for each diet/exposure with exception of low Fe diet/vehicle exposure, where only 1 sample was available; Suppl. Fig. 1).

2.8. Statistical analysis

Acute mortality immediately preceding the 24 h. MeHg or vehicle exposure was compared across treatments using the Chi-square test. Lifespan and maturation time were compared across treatments using the Kaplan-Meier distribution and Wilcoxon rank sum test. Missing samples or accidental deaths ($N=4$) were censored from the lifespan analysis. Algal and *Daphnia* Fe concentrations are compared using one-way ANOVA or t-test, while reproductive parameters (total reproduction and average brood size) are compared across treatment and diet using a two-way ANOVA (JMP 10, fit model platform, standard least

squares fit, factorial macro). For analysis of % storage lipids from ORO stained *Daphnia*, a multivariate linear regression model was used to estimate the effects of diet, exposure to MeHg and/or vehicle, and potential modifying effects of total body area, number of *Daphnia* in jars (independent variables), on % lipid (dependent variable). A nested ANOVA (JMP 10, fit model platform, restricted maximum likelihood or REML) was used to analyze TAG concentrations, treating replicate measurements from biological samples as a random effect nested within diet and exposure (fixed effects) (Krzywinski et al., 2014). Analyses were conducted in JMP 10. All statistics were deemed statistically significant when $P \leq 0.05$.

3. Results

3.1. Iron quantification in *A. falcatus* diet and *D. pulex*

A reduced Fe diet for *Daphnia* was produced by culturing *A. falcatus* in standard, 50%, 10% and no Fe media (3.15, 1.57, 0.315, and 0 mg FeCl₃/L). After harvesting and rinsing, the Fe content of *A. falcatus* grown in various Fe medium was measured. The mean Fe concentration of *A. falcatus* grown in the standard Fe growth media (28.84 ± 0.44 mg Fe/100,000 cells) contains about twice the amount measured in 50% Fe media (14.89 ± 0.14 mg Fe/100,000 cells) while algae grown in 10% and no Fe media had even lower Fe concentrations (0.43 ± 0.02 and 0.10 ± 0.02 mg Fe/100,000 cells, respectively). The mean Fe concentrations of the algae were significantly different from each other ($p < 0.0001$) except when comparing between the 10% and no Fe algal Fe concentrations ($p = 0.35$) (Fig. 1A).

After the *Daphnia* were fed a standard or 50% Fe diet, the Fe concentration in *Daphnia* was measured. Fe levels of *D. pulex* on the 50% Fe diet were significantly lower compared to those fed a standard Fe diet (means = 8.40 ± 1.29 μ g vs. 21.95 ± 3.83 μ g Fe/*D. pulex*; $p = 0.015$) which suggests that the Fe content in *Daphnia* significantly decreases when fed algae that was grown in media with reduced Fe (Fig. 1B).

3.2. Lifespan

There was a significant decline in the mean lifespan of *D. pulex* (39.8 ± 1.2 , 36.6 ± 1.2 , 28.6 ± 1.4 , 29.8 ± 1.2 days for standard, 50%, 10%, and no Fe, respectively) as Fe was reduced in the diet (Wilcoxon Rank Sum $p < 0.05$; see Table 1 and Fig. 2A).

Daphnia on standard and 50% Fe diets were exposed to MeHg (1600 ng/L) for 24 h in the first 48 h of life, then housed separately after exposure to track individual reproduction and lifespan. There was a significant difference in acute mortality immediately following the early life exposure among the treatment groups (Pearson's Chi square = 21.57; $p < 0.0001$) explained by a significant effect of a low Fe diet (MeHg: Pearson's Chi square = 11.08; $p = 0.0009$; Vehicle: Pearson's Chi square = 10.35; $p = 0.0013$) (Table 2). *D. pulex* that were fed a low Fe diet or exposed to MeHg early in life did not have a significant difference in lifespan compared across treatment groups except when compared between low Fe *Daphnia* exposed to vehicle and MeHg exposed *Daphnia* on a standard Fe diet (Table 3).

3.3. Maturation time (time to first reproduction)

For *Daphnia* fed the standard, 50%, 10%, and no Fe diets, the mean number of days until first reproduction increased with decreasing Fe in diet (8.4 ± 0.7 , 9.5 ± 0.8 , 10.5 ± 1.7 , 11.0 ± 1.2 days for standard, 50%, 10%, and no Fe, respectively) (Fig. 2B); however, there was no significant difference between any group (ANOVA: $p = 0.31$).

Daphnia on standard and 50% Fe diets and exposed to either vehicle or MeHg (1600 ng/L) significantly delayed the time to first reproduction compared to the no exposure groups in standard and low Fe diets (Low Fe: 10.0 ± 0.3 , 11.5 ± 0.3 , 11.6 ± 0.3 ; Standard Fe: 10.9 ± 0.5 , 11.7 ± 0.3 , 11.6 ± 0.3 for control, vehicle, and MeHg, respectively). MeHg and vehicle controls were not significantly different from each other when compared in standard and/or low Fe diets (Table 4).

3.4. Average brood size and reproduction rate

For *Daphnia* on standard and 50% Fe diets that were exposed to MeHg in early life, the average brood size for each treatment were approximately 5 offspring per clutch (No exposure: 5.0 ± 0.3 , 5.3 ± 0.2 ; Vehicle: 4.8 ± 0.2 , 5.3 ± 0.3 ; MeHg: 5.3 ± 0.3 , 5.3 ± 0.2 offspring/clutch for standard and low Fe, respectively) while the mean reproduction rate for each group ranges between 1 and 2 *Daphnia* each day (No exposure: 1.7 ± 0.1 , 1.7 ± 0.1 ; Vehicle: 1.6 ± 0.1 , 1.6 ± 0.2 ; MeHg: 1.7 ± 0.1 , 1.6 ± 0.1 offspring/day for standard and low Fe, respectively). The average brood size and overall reproduction rate were not significantly different in MeHg exposed *Daphnia* (Average brood size: $p = 0.54$; Reproduction rate: $p = 0.79$) or for *Daphnia* on a low Fe diet (Average brood size: $p = 0.23$; Reproduction rate: $p = 0.66$). Furthermore, the interaction of exposure and diet did not show a significant effect on average brood size or reproduction rate (Average brood size: $p = 0.65$; Reproduction rate: $p = 0.90$). From this, one can conclude that neither diet nor MeHg exposure affected the average brood size or reproduction rate in this sample of *D. pulex*.

3.5. Lipid reserves

A total of 149 ORO-stained 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 left a total of 141 images for statistical analysis. The low Fe no exposure group had the lowest mean % lipid (0.98% lipid) while *Daphnia* fed the low Fe diet and exposed to MeHg early in life had the highest mean lipid percentage (4.6% lipid). There is a notable trend in the mean of different exposures, with lipid percentages increased in MeHg exposed compared to controls in each diet (Standard Fe: 2.9 ± 0.3 , 2.2 ± 0.3 , 4.0 ± 0.3 ; Low Fe: 1.9 ± 0.3 , 1.0 ± 0.2 , 4.6 ± 0.6 for no exposure, vehicle, and MeHg, respectively). Representative images for each diet and MeHg exposure are shown in Fig. 3. Since increased body size is associated with a heightened % lipid in *Daphnia*, we adjusted for total body area. In addition, the amount of *Daphnia* in jars was adjusted for in multivariate linear regression analyses. Model 1, summarized in Table 5, suggests that the exposure regime (to vehicle alone or MeHg + vehicle), and the interaction between diet and MeHg have a significant effect on the lipid/body percentage in *D. pulex*. Removing non-significant terms (diet, # of *Daphnia* in jars, and diet*vehicle interaction term) further suggests that MeHg and a low Fe diet may interact to increase lipid/body

percentage; however goodness of fit parameters between the 2 models are not significantly different (Akaike Information Criterion (AIC) reduced from 503 to 500) (Table 5). Results from the TAG quantification assay are consistent with ORO results in that *Daphnia* that were exposed to vehicle alone or MeHg + vehicle show an increase in TAG concentration compared to controls (Suppl. Table 1 and Suppl. Fig. 1, nested ANOVA exposure $p = 0.03$) whereas *Daphnia* on the low Fe diet had non-significant heightened TAG levels compared to those on the standard diet (Suppl. Table 1 and Suppl. Fig. 1, nested ANOVA $p = 0.13$). Unlike the ORO results, TAG measurements do not indicate an interaction between MeHg exposure and diet ($p = 0.60$).

4. Discussion

The presented research uses *D. pulex* to investigate the interaction between early-life MeHg exposure and a low-Fe diet. To our knowledge, this is the first study that has investigated the effects of low-Fe diets in *D. pulex*, which is relevant to development of alternative screening models of ID, a common and costly condition in human populations. As in mammals, Fe is essential for *Daphnia* in hemoglobin synthesis and reproduction (Engle, 1985; Smaridge, 1956; Dave, 1984). Indeed, past studies indicate that ID or low Fe levels increase the risk of mortality in humans and rodent models (Gambling et al., 2003; Klip et al., 2013; Dave, 1984), but these effects may be subtle and long-term as Teilsch et al. (2006) found Fe levels had no significant association with childhood mortality.

We have shown that low Fe diets can be developed by growing algae in low Fe media and when used as a food source for *D. pulex* result in reduced Fe levels in *D. pulex* tissue. Data from this study showed a significant decrease in lifespan and reduced (but non-significant) maturation rate in *Daphnia* fed reduced Fe diets, indicating further research with larger sample sizes may be required.

Another purpose of this study was to evaluate a multi-stressor hypothesis relevant to human exposures across the lifespan. Past studies in humans and rodent models indicate that ID and MeHg exposure is associated with dyslipidemia (Nandyala et al., 2013; Stangl and Kirchgessner, 1998; Moreira et al., 2012). Previous studies in *Caenorhabditis elegans* and *Drosophila melanogaster* have demonstrated the utility of invertebrate model systems for understanding dysregulation of lipid metabolism in humans (Dourlen et al., 2015). The present study develops a method to stain and quantify lipid reserves in *Daphnia* using Oil Red O followed by image analysis. Interestingly, results from the present analysis suggest 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 increase in lipid storage.

In the current experiment, *Daphnia* underwent an acute (24hr.) exposure to MeHg to investigate adverse long-term effects; therefore this exposure paradigm may have limited generalizability to chronic exposure to MeHg. 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 and McNaught, 1992). In this study, there were no differences in the reproduction rate when *Daphnia* were subjected to an acute 24 h exposure to 1600 ng/L MeHgCl, although a previous study detected a reduced lifespan and decreased reproduction

after this exposure (Doke et al., 2014). We believe this inconsistency between the current results and previous results could be due to diet variation since different algae batches were used in each experiment. Although algal cell number and cell size is accounted for, there may have been differences in macro and micronutrient content and algal defenses that decrease digestibility due to highly dynamic growth/degradation processes (DeMott and Van Donk, 2013; Talukdar et al., 2012). Going forward, standardized feeding techniques will be critical for determining reliability of this system. In addition, the increased acute mortality and a delay in the start of reproduction evident in both MeHg and vehicle only exposures, suggests significant stress is caused by the additional handling and/or 24 h without food.

In the present analysis, results from the quantification of triacylglycerol (TAG) using an enzymatic assay kit were somewhat consistent with ORO image analysis, however significant variation across biological replicates existed. This finding differs from past studies, suggesting that lipids stained with ORO did correspond to the amount of lipids quantified with biochemical assays in rodent tissue or cell cultures (Ramírez-Zacarias et al., 1992; Ge et al., 2010). Based on the present analysis, we suggest using commercial kits for quantifying lipids in *D. pulex* may not be 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 highly correlated with the body size of *D. pulex*, which is consistent 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.) since high background glycerol content was observed in the wells, commercial assays dependent on conversion of TAG to glycerol is not optimal, as has been shown in *Drosophila*, another invertebrate model system (Hildebrandt et al., 2011). The lipid content of the zooplankton is affected by its overall food quality (Ahlgren et al., 1990; Weers and Gulati, 1997); therefore the lipid content of the algae grown in the Fe-depleted media cannot be ruled out as a possible explanation of the current results; however based on previous results showing decreased lipid content in algae grown in Fe-depleted media (Fan et al., 2014), we would not expect the present finding of a slight increase in lipid content in *Daphnia* fed a low Fe diet.

5. Conclusion

This present analysis demonstrates *D. pulex* may be a useful alternative whole animal model to test multi-stressor interactions across the lifespan, complimenting current *in vitro* screening methods such as 3-D cell systems, which cannot evaluate trans-generational or long-term, integrative effects across organ systems and over a lifespan. While initial experiments suggest a unique and complementary role for this model system in toxicity screening, standardization of culturing techniques and systematic evaluation of reproducibility within and across labs maintaining clonal populations will be important next steps. Additional research examining the effects of dietary Fe concentrations in *Daphnia* may aid in elucidation of subtle effects on long-term outcomes related to reproduction, development, lipid storage and lifespan.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CDC	Center for Disease Control and Prevention
EDTA	ethyl-enediaminetetraacetic acid
Fe	iron
ID	iron deficiency
MeHg	methylmercury
MeOH	methanol
NIH	National Institutes of Health
ORO	Oil Red O
SEM	standard error of the mean
TAG	triacylglycerol
WHO	World Health Organization

References

- Ahlgren G, Lundstedt L, Brett M, Forsberg C. Lipid composition and food quality of some freshwater phytoplankton for cladoceran zooplankters. *J. Plankton Res.* 1990; 12(4):809–818.
- Arzate-Cárdenas MA, Martínez-Jerónimo F. Energy reserve modification in different age groups of *Daphnia schoedleri* (Anomopoda: Daphniidae) exposed to hexavalent chromium. *Environ. Toxicol. Pharmacol.* 2012; 34:106–116. [PubMed: 22481114]
- Askwith C, Kaplan J. Iron and copper transport in yeast and its relevance to human disease. *Trends Biochem. Sci.* 1998; 23(4):135–138. [PubMed: 9584616]
- Asselman J, Shaw JR, Glaholt SP, Colbourne JK, De Schampelaere KA. Transcription patterns of genes encoding four metallothionein homologs in *Daphnia pulex* exposed to copper and cadmium are time- and homolog-dependent. *Aquat. Toxicol.* 2013; 142:422–430. [PubMed: 24113165]

- Bošnjir J, Puntari D, Cvetkovi Ž, Pollak L, Barušić L, Klarić I, Miškulin M, Puntarić I, Puntarić E, Milošević M. Effects of magnesium, chromium, iron and zinc from food supplements on selected aquatic organisms. *Coll. Antropol.* 2013; 37(3):965–971. [PubMed: 24308244]
- Brabin L, Brabin BJ, Gies S. Influence of iron status on risk of maternal or neonatal infection and on neonatal mortality with an emphasis on developing countries. *Nutr. Rev.* 2013; 71(8):528–540. [PubMed: 23865798]
- Centers for Disease Control and Prevention. Iron deficiency-United States 1999–2000. *Morb. Mortal. Wkly. Rep.* 2002; 51(40):897–899.
- Colbourne JK, Pfrender ME, Gilbert D, Thomas WK, Tucker A, Oakley TH, Tsuchiya D. The ecoresponsive genome of *Daphnia pulex*. *Science*. 2011; 331(6017):555–561. [PubMed: 21292972]
- Dave G. Effects of waterborne iron on growth, reproduction, survival and haemoglobin in *Daphnia magna*. *Comp. Biochem. Physiol. Part C Comp. Pharmacol.* 1984; 78(2):433–438.
- Davidson PW, Strain JJ, Myers GJ, Thurston SW, Bonham MP, Shamlaye CF, Clarkson TW. Neurodevelopmental effects of maternal nutritional status and exposure to methylmercury from eating fish during pregnancy. *Neurotoxicology*. 2008; 29(5):767–775. [PubMed: 18590763]
- DeCoen WM, Janssen CR. The missing biomarker link: relationships between effects on the cellular energy allocation biomarker of toxicant-stressed *Daphnia magna* and corresponding population characteristics. *Environ. Toxicol. Chem.* 2002; 22(7):1632–1641.
- DeMott WR, Van Donk E. Strong interactions between stoichiometric constraints and algal defenses: evidence from population dynamics of *Daphnia* and algae in phosphorus-limited microcosms. *Oecologia*. 2013; 171:175–186. [PubMed: 22802021]
- Deutsch MJ, Schriever SC, Roscher AA, Ensenuer R. Digital image analysis approach of lipid droplet size quantitation of oil-red-o stained cultured cells. *Anal. Biochem.* 2014; 445:87–89. [PubMed: 24120410]
- Doke DA, Hudson SL, Dawson JA, Gohlke JM. Effects of early life exposure to methylmercury in *Daphnia pulex* on standard and reduced food ration. *Reprod. Toxicol.* 2014; 49:219–225. [PubMed: 25263226]
- Dourlen P, Sujkowski A, Wessells R, Mollereau B. Fatty acid transport proteins in disease: new insights from invertebrate models. *Prog. Lipid Res.* 2015 <http://dx.doi.org/10.1016/j.plipres.2015.08.001>.
- Engle D. The production of haemoglobin by small pond *Daphnia pulex*: intraspecific variation and its relation to habitat. *Freshw. Biol.* 1985; 15:631–638.
- Fan J, Cui Y, Wan M, Wang W, Li Y. Lipid accumulation and biosynthesis genes response of the oleaginous *Chlorella pyrenoidosa* under three nutrition stressors. *Biotechnol. Biofuels*. 2014; 7:7–17. [PubMed: 24405789]
- Fonseca, MdF; Hacon, SDS.; Grandjean, P.; Choi, AL.; Bastos, WR. Iron status as a covariate in methylmercury-associated neurotoxicity risk. *Chemosphere*. 2014; 100:89–96. [PubMed: 24411835]
- Gambling L, Dunford S, Wallace DI, Zuur G, Solanky N, Srail SKS, McArdle HJ. Iron deficiency during pregnancy affects postnatal blood pressure in the rat. *J. Physiol.* 2003; 552(2):603–610. [PubMed: 14561840]
- García-Casal MN, Pereira AC, Leets I, Ramírez J, Quiroga MF. High iron content and bioavailability in humans from four species of marine algae. *J. Nutr.* 2007; 137:2691–2695. [PubMed: 18029485]
- Ge F, Lobdell H, Zhou S, Hu C, Berk PD. Digital analysis of hepatic sections in mice accurately quantitates triglycerides and selected properties of lipid droplets. *Exp. Biol. Med.* 2010; 235(11):1282–1286.
- Goulden CE, Place AR. Fatty acid synthesis and accumulation rates in daphniids. *J. Exp. Zool.* 1990; 256(2):168–178.
- Hildebrandt A, Bickmeyer I, Kühnlein RP. Reliable *Drosophila* body fat quantification by a coupled colorimetric assay. *PLoS One*. 2011; 6(9):e23796. <http://dx.doi.org/10.1371/journal.pone.0023796>. [PubMed: 21931614]
- Karagas M, Choi AL, Oken E, Horvat M, Schoeny R, Kamai E, Korrick S. Evidence on the human health effects of low level methylmercury exposure. *Environ. Health Perspect.* 2012; 120(6):799–806. [PubMed: 22275730]

- Kilham SS, Kreeger DA, Goulden CE, Lynn SG. Effects of algal food quality on fecundity and population growth rates of daphnia. *Freshw. Biol.* 1997; 38:639–647.
- Kilham SS, Kreeger DA, Lynn SG, Goulden CE, Herrera L. Combo: a defined freshwater culture medium for algae and zooplankton. *Hydrobiologia.* 1998; 377:147–159.
- Klip IT, Comin-Colet J, Voors AA, Ponikowski P, Enjuanes C, Banasiak W, Lok DJ, Rosentryt P, Torrens A, Polonski L, van Veldhuisen DJ, van der Meer P, Jankowska EA. Iron deficiency in chronic heart failure: an international pooled analysis. *Am. Heart J.* 2013; 165:575–582. [PubMed: 23537975]
- Kosakowska A, Lewandowska J, Sto J, Burkiewicz K. Qualitative and quantitative composition of pigments in *Phaeodactylum tricornutum* (Bacillariophyceae) stressed by iron. *BioMetals.* 2004; 17:45–52. [PubMed: 14977361]
- Krzywinski M, Altman N, Blainey P. Nested designs. *Nat. Methods.* 2014; 11(10):977–978. [PubMed: 25392877]
- Lampert W. Daphnia: model herbivore, predator, and prey. *Pol. J. Ecol.* 2006; 54(4):607–620.
- LeBlanc G. Acute toxicity of priority pollutants to water flea (*Daphnia magna*). *Bull. Environ. Contam. Toxicol.* 1980; 24(1):684–691.
- Li YQ, Cao XX, Bai B, Zhang JN, Wang MQ. Severe iron deficiency is associated with a reduced conception rate in female rats. *Gynecol. Obstet. Investig.* 2014; 77:19–23. [PubMed: 24134949]
- Moreira EL, de Oliveira J, Dutra MF, Santos DB, Gonçalves CA, Goldfeder EM, Farina M. Does methylmercury-induced hypercholesterolemia play a causal role in its neurotoxicity and cardiovascular disease? *Toxicol. Sci.* 2012; 130(2):373. [PubMed: 22903822]
- National Research Council (NRC). Committee on Toxicity Testing and Assessment of Environmental Agents. Washington, D.C: National Academies Press; 2007. *Toxicity Testing in the 21st Century: a Vision and a Strategy*; p. 216 ISBN 978-0-309-10988-8
- National Institutes of Health. Daphnia. Model Organisms for Biomedical Research. 2013. www.nih.gov/science/models/daphnia
- Nandyala V, Gandiah P, Karthik RS, Sivarajappa P, Indira G, Krishna Prasad T. A study of lipid profile in iron deficiency anemia. *Int. J. Recent Trends Sci. Technol.* 2013; 9(2):258–266.
- Radlowski EC, Johnson RW. Perinatal iron deficiency and neurocognitive development. *Front. Hum. Neurosci.* 2013; 7:585. [PubMed: 24065908]
- Ramírez-Zacarias JL, Castro-Muñozledo F, Kuri-Harcuch W. Quantification of adipose conversion and triglycerides by staining intracytoplasmic lipids with Oil Red O. *Histochemistry.* 1992; 97:493–497. [PubMed: 1385366]
- Rao J, Jagadeesan V. Development of a rat model for iron deficiency and toxicological studies: comparison among Fischer 344, Wistar, and Sprague Dawley strains. *Lab. Anim. Sci.* 1995; 45(4):393–397. [PubMed: 7474878]
- Rasband, WS. ImageJ. Bethesda, Maryland, USA: U. S. National Institutes of Health; 1997–2014. <http://imagej.nih.gov/ij/>
- Rovida C, Asakura S, Daneshian M, Hofman-Huether H, Leist M, Meunier L, Reif D, Rossi A, Schmutz M, Hartung T. *Toxicity Testing in the 21st Century Beyond Environmental Chemicals.* 2015
- Schwartz SS, Hebert PD. Methods for the activation of the resting eggs of *Daphnia*. *Freshw. Biol.* 1987; 17:373–379.
- Shaw JR, Colbourne JK, Davey JC, Glaholt SP, Hampton TH, Chen CY, Folt CL, Hamilton JW. Gene response profiles for *Daphnia pulex* exposed to the environmental stressor cadmium reveals novel crustacean metallothioneins. *BMC Genom.* 2007; 8:477.
- Shaw JR, Pfrender ME, Eads BD, Klaper R, Callaghan A, Sibly RM, Colson I, Jansen B, Golbert D, Colbourne JK. *Daphnia* as an emerging model for toxicological genomics. *Adv. Exp. Biol.* 2008; 2:165–328.
- Sherman AR. Serum lipids in suckling and post-weaning iron-deficient rats. *Lipids.* 1979; 14(11):888–892. [PubMed: 513975]
- Smaridge MW. Distribution of iron in daphnia in relation to haemoglobin synthesis and breakdown. *Q. J. Microsc. Sci.* 1956; 97(2):205–214.

- Soukas AA, Kane EA, Carr CE, Melo JA, Ruvkun G. Rictor/TORC2 regulates fat metabolism, feeding, growth, and life span in *Caenorhabditis elegans*. *Genes Dev.* 2009; 23:496–511. [PubMed: 19240135]
- Spitze K. Population structure in *Daphnia obtusa*: quantitative genetic and allozymic variation. *Genetics.* 1993; 135:367–374. [PubMed: 8244001]
- Stamps WT, Linit MJ. A rapid and simple method for staining lipid in fixed nematodes. *J. Nematol.* 1995; 27(2):244–247. [PubMed: 19277286]
- Stangl GI, Kirchgessner M. Different degrees of moderate iron deficiency modulate lipid metabolism of rats. *Lipids.* 1998; 33:889–895. [PubMed: 9778136]
- Stern AH. A review of the studies of the cardiovascular health effects of methylmercury with consideration of their suitability for risk assessment. *Environ. Res.* 2005; 98(1):133–142. [PubMed: 15721894]
- Talukdar J, Kalita MC, Goswami BC. Influence of dissolved inorganic carbon and nitrogen sources on growth, total lipid content and calorific value of the freshwater oleaginous microalgae *Ankistrodesmus falcatus* (Corda) Ralfs. *Environ. Res. Eng. Manag.* 2012; 3(61):14–25.
- Tessier AJ, Goulden CE. Estimating food limitation in cladoceran populations. *Limnol. Oceanogr.* 1982; 27(4):707–717.
- Tian-yi C, McNaught DC. Toxicity of methylmercury to *Daphnia pulex*. *Bull. Environ. Contam. Toxicol.* 1992; 49(4):606–612. [PubMed: 1421856]
- Teilsch JM, Khatri SK, Stoltzfus RJ, Katz J, LeClerq SC, Adhikari R, Mullany LC, Shrestha S, Black RE. Effect of routine prophylactic supplementation with iron and folic acid on preschool child mortality in southern Nepal: community-based, cluster- randomised, placebo-controlled trial. *Lancet.* 2006; 367(9505):144–152. [PubMed: 16413878]
- Weers PMM, Gulati RD. Growth and reproduction of *Daphnia galeata* in response to changes in fatty acids, phosphorus, and nitrogen in *Chlamydomonas reinhardtii*. *Limnol. Oceanogr.* 1997; 42(7): 1584–1589.
- Yen K, Le TT, Bansal A, Narasimhan SD, Cheng JX, Tissenbaum HA. A comparative study of fat storage quantitation in nematode *Caenorhabditis elegans* using label and label-free methods. *PLoS One.* 2010; 5(9):e12810. <http://dx.doi.org/10.1371/journal.pone.0012810>. [PubMed: 20862331]
- Yu GS, Steinkirchner TM, Rao GA, Larkin EC. Effect of prenatal iron deficiency on myelination in rat pups. *Am. J. Pathol.* 1986; 125(3):620–624. [PubMed: 2432794]
- Zimmermann MB, Hurrell RF. Nutritional iron deficiency. *Lancet.* 2007; 370:511–520. [PubMed: 17693180]

Fig. 1.

Iron concentrations in algal food source and *D. pulex*. The concentration of Fe in (A) Algae *Ankistrodesmus falcatus* grown in Standard, 50%, 10% and No Fe media (n = 3 in each group) and (B) *Daphnia pulex* fed Standard and 50% Fe diets (n = 4 in each group). Values represent mean $\pm$ standard error of the mean (SEM).

Fig. 2.

The effect of a low iron diet on lifespan and time to first reproduction in *D. pulex*. (A) Survival across the lifespan and (B) age to first reproduction in standard and reduced Fe diets. Values represent mean $\pm$ standard error of the mean (SEM). Based on survival analysis, there are significant differences in lifespan between all groups except when comparing 10% and no Fe diets. Age to first reproduction is not significantly different between groups (ANOVA: $p = 0.31$).

Fig. 3.

Representative images of *D. pulex* stained with Oil Red O. Neutral lipids in Daphnia were stained with Oil Red O (bright red). Percent lipids of total body area estimated for each image shown: Standard Fe: (A) No exposure: 2.17% (B) MeHg: 4.20%; Low Fe: (C) No Exposure: 0.65% and (D) MeHg: 4.59%. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1

Wilcoxon test results for lifespan in *D. pulex* on different Fe diets.

Diet	50% Fe	10% Fe	No Fe
Standard Fe	0.0496 ^a	<.0001 ^a	<.0001 ^a
50% Fe		<.0001 ^a	<.0001 ^a
10% Fe			0.4850

^aIndicates statistical significance.

Table 2Acute mortality in *D. pulex* 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

Table 3

Lifespan in *D. pulex* across diet and MeHg exposure.

Diet	Treatment (ng MeHg/L)	N=	Mean lifespan $\pm$ SEM (Days)	Wilcoxon rank sum p-values			
				Low Fe		Standard Fe	
				No exposure	Vehicle	No exposure	Vehicle
Low Fe	MeHg (1600)	20	43.7 $\pm$ 2.6	0.2363	0.0651	0.4757	0.7971
Low Fe	No exposure (0)	20	35.4 $\pm$ 5.1		0.5062	0.1354	0.3866
Low Fe	Vehicle (0)	19	30.4 $\pm$ 4.7			0.0093 ^a	0.0966
Standard Fe	MeHg (1600)	19	44.7 $\pm$ 3.6				0.6269
Standard Fe	No exposure (0)	20	41.9 $\pm$ 4.8				0.0861
Standard Fe	Vehicle (0)	18	35.7 $\pm$ 5.5				0.3117

^aIndicates statistical significance.

Table 4

Time to first reproduction in *D. pulex* across diet and MeHg exposure.

Diet	Treatment (ng MeHg/L)	N	Mean ± SEM (Days)	Wilcoxon rank sum p-values			
				Low Fe		Standard Fe	
				No exposure	Vehicle	1600 ng/L	No exposure
Low Fe	MeHg (1600)	19	11.6 ± 0.3	<0.0001 ^a	0.8779	0.9453	0.0014 ^a
Low Fe	No exposure (0)	15	10.0 ± 0.3		<0.0001 ^a	<0.0001 ^a	0.1966
Low Fe	Vehicle (0)	12	11.5 ± 0.3			0.9453	0.0083 ^a
Standard Fe	MeHg (1600)	19	11.6 ± 0.3				0.0014 ^a
Standard Fe	No exposure (0)	16	10.9 ± 0.5				0.8148
Standard Fe	Vehicle (0)	12	11.7 ± 0.3				0.0056 ^a

^aIndicates statistical significance.

Table 5

Multivariate regression model for % lipids and its covariates.

Model	Covariate	Estimate	Std error	t Ratio	Prob> t	AIC
1	Diet (Low Fe)	-0.05	0.17	-0.30	0.7627	503
	Vehicle (No Vehicle)	-0.88	0.17	-5.29	<.0001 ^a	
	MeHg (No MeHg)	-0.32	0.25	-1.30	0.1944	
	# of Daphnia in jars	0.01	0.01	0.17	0.8674	
	Total body area	4.78	0.66	7.21	<.0001 ^a	
2	Diet* MeHg (Low Fe* No MeHg)	-0.38	0.15	-2.50	0.0135 ^a	500
	Diet* vehicle (Low Fe* No Vehicle)	0.18	0.14	1.28	0.2036	
	Vehicle (No Vehicle)	-0.88	0.15	-5.85	<.0001 ^a	
	MeHg (No MeHg)	-0.38	0.17	-2.27	0.0250 ^a	
	Total body area	4.67	0.57	8.25	<.0001 ^a	
	Diet* MeHg (Low Fe* No MeHg)	-0.40	0.12	-3.38	0.0009 ^a	

^aIndicates statistical significance.