EFFECTS OF EARLY LIFE EXPOSURE TO METHYLMERCURY ON PREDATOR RESPONSE IN *Daphnia pulex*

by

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ENVIRONMENTAL HEALTH AND TOXICOLOGY

ABSTRACT

Knowledge of the health effects of toxic chemicals is largely based on single chemical models rather than a multi-factor model, which more accurately captures real-world exposures. This study investigated how methylmercury affects *Daphnia pulex* in the presence of predatory stress chemical. A kairomone is an infochemical released by an animal that can be detected by another animal. *Daphnia pulex* detects kairomone released by *Danio rerio* resulting in the induction of morphological and life history changes. *Danio rerio* were kept in COMBO media for 24 hours at a density of 2 fish per liter. Daphnids were then housed in kairomone-containing COMBO for their full life cycle. Following this kairomone exposure, adult females were additionally exposed to 1600 ng/L of methylmercury for 24 hours in the absence of food. Female offspring were collected every 48 hours and measurements were taken of tail-spine and body length (central body including head) as well as soma width to determine the impact of the combined exposure on these morphological end-points. Life history traits observed included life span and brood size. Our data shows that maternal exposure to methylmercury had a significant negative impact on the predatory responses including tail spine and body size (length and width) in offspring two days old or less. Brood size was not significantly impacted in this system; however, life span was significantly lessened. These data demonstrate the necessity of using multi-factor models in testing the toxicity of environmental exposures in bio-sentinels.
Keywords: methylmercury, kairomone, Daphnia, toxicology, environment
DEDICATION

I dedicate all my hard work to my family but especially my husband, Tim. Without your encouraging words and help at 2 am I don’t think I could have finished this project.
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LIST OF ABREVIATIONS

ADHD – Attention Deficit Hyperactivity Disorder
ADPH – Alabama Department of Public Health
ATSDR - Agency for Toxic Substances and Disease Registry
DHA – Docosahexaenoic acid
EPA – Eicosapentaenoic acid
IACUC - Institutional Animal Care and Use Committee
JHAMT – Juvenile Hormone Acid Methyltransferase
MeHg – Methylmercury
MET – Methoprene-tolerant
NIH - National Institutes of Health
OECD - Organization for Economic Co-operation and Development
PVC – Poly vinyl-chloride
TAG – Triglyceride
UNEP – United Nations Environment Programme
USEPA – United States Environmental Protection Agency
INTRODUCTION

Knowledge of the health effects of toxic chemicals are largely based on single chemical models that do not introduce any additional stress into the model. While this provides vital knowledge about individual toxic chemicals, it may not tell the whole story. It is not likely that a person will be exposed to a single chemical at a time with no additional stress in their daily lives. This study sought a more real world look at the effects of methylmercury by using predator threat as an additional stress. Predator threat can be translated into a human stress by viewing it as simply an intimidating situation such as a competitive job market or stressful home life.

Daphnia

*Daphnia* is a genus of crustacean zooplankton that is approximately 3.5 mm in length and which are found in most permanent bodies of nutrient-rich fresh water (Miller, 2000). Of the more than 100 species (Ebert, 2005) found in the northern hemisphere (US EPA, 2002) worldwide, the most common species is *D. pulex* (Miller, 2000). *Daphnia* are crucial members of the freshwater ecosystem. *D. pulex* is a filter feeder that primarily consumes algae that it encounters in the water. While algae are the primary source of food, *Daphnia* also consume bacteria and fine detritus (Miller, 2000). *Daphnia* are an important part of the freshwater food chain, being preyed upon by fish spp., *Chaoborus* spp., and *Notonecta* spp. (Spitze and Sadler, 1996; Kreuger and Dodson, 1981, Dodson 1989; Hanazato et al., 2001).
While *D. pulex* is geographically widespread in the northern hemisphere, there is little variation within a given population due to parthenogenic reproduction (US EPA, 2002). Sexual reproduction is possible within the species with the production of males and resting eggs during times of stress, including lower temperatures, heavy population densities, food shortages, and other environmental factors that threaten survival.

*Daphnia* is sensitive to environmental changes and is commonly used in ecotoxicological studies (NIH). *D. pulex* has been accepted as a toxicological model by the National Institutes of Health, US Environmental Protection Agency (USEPA), and Organization for Economic Co-operation and Development (OECD). *Daphnia* have several characteristics that make them a useful species for environmental toxicological research; these include their short lifespan (approximately 50 days (US EPA, 2002)), parthenogenic reproduction (avoids issues of variable genetic backgrounds), the production of relatively large numbers of offspring over a lifetime (high fecundity), and that they are relatively inexpensive to rear in the laboratory.

*D. pulex* go through four developmental stages in its approximately 50 day life span. The egg is considered the first stage of life. The US EPA considers the start of life as the time the egg is released into the brood chamber of the adult daphnid. Eggs hatch within the brood chamber of the adult and offspring that are released directly before the next molt are born into the juvenile stage, the second stage. The adolescent or third stage occurs when the first egg clutch is released into the brood chamber and ends at the birth of the first brood, after-which the daphnid enters the adult stage. At this fourth and final stage, the daphnid releases a brood typically every 2 days until the end of its life.
Many organisms use infochemicals to signal to other organisms both within their own species as well as across species. These infochemicals can be used by the organism to attract another species with which it has evolved to carry on a symbiotic, mutualistic or commensal relationship. One example of this type of interaction would be a plant that has been attacked by a caterpillar releasing an infochemical that attracts a parasitic wasp, such as *Microplitis croceipes*, that then in turn attack the caterpillar (Turlings *et al.*, 1995). The infochemical released by many aquatic organisms is referred to as a kairomone (Petrusek *et al.*, 2009). “A kairomone is a heat-stable, nondialyzable, pronase-sensitive factor that is probably a protein” (Tollrian and Harvel, 1999). More generally, a kairomone can be any allelochemicals released by one species that can be picked up by another, including hormones, that benefits the receiving species (Allison *et al.*, 2001, Meyer, 2006). An allelochemical can benefit the releasing species, the receiving species, or both. If the allelochemical benefits only the releasing species then it is known as an allomone. If the allelochemical only benefits the receiving species then it is a kairomone. When both species benefit from the released allelochemical it is called a synomone (Meyer, 2006). Prey animals (such as Daphnia) can pick up on these chemicals and use them as a signal to begin efforts to avoid predation (Petrusek *et al.*, 2009).

**Methylmercury**

Mercury is a heavy metal that is readily available in multiple forms in the environment. Elemental mercury and organic mercury (methylmercury) are common
forms of mercury that can be found in the environment (USGS, 2000). Elemental mercury is harmful when inhaled (mercury vapor) which may occur in certain occupational settings including electrical equipment and automotive part manufacture and dental professions (ATSDR, 1999). Methylmercury is toxic to humans when blood concentrations are at an elevated level. Methylmercury can be transported across the blood brain barrier (BBB) within 4 hours of ingestion via MeHg-L-Cys complex (Kerper et al., 1992). The most sensitive populations are unborn children as well as young children whose brains are not fully developed at the time of the exposure. Consumption of contaminated fish is the typical exposure route; however it is known that fish consumption can also have significant health benefits. This leads to the paradigm of how much consumption is too much for a particular species of fish, to determine the relative risks from the deleterious methylmercury and the beneficial properties. Consuming 1-2 servings a week of fish species high in n-3 fatty acids eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) has health benefits including a reduced risk for coronary heart disease and sudden coronary related death (Mozaffarian and Rimm, 2006). Consumption of oily fish such as salmon, herring, and sardines appears to reduce risk more so than less oily species of fish such as catfish, cod, and halibut. Not only are salmon and sardines healthy due to higher concentrations of EPA and DHA but these species of fish are relatively short lived leading to a lower concentration of methylmercury in the tissue of the fish as it doesn’t have time to bioaccumulate.

In the United States, fish consumption advisories are issued for any body of water that is found to contain fish with elevated levels of methylmercury within the tissue of the sampled fish. To go beyond this, states publish and distribute a guide to aid in the
selection and quantities of fish that are consumed, with one example illustrated in Figure 1A. In order to reduce the risks associated with fish consumption from waterways in which an advisory has been issued, preparation for cooking should include the removal of all fatty tissue, skin, and internal organs, and only the filet should be cooked and consumed (Alabama Department of Public Health (ADPH), 2007). The ADPH also suggests that all fish be cooked in a manner that allows the fat to drip away from the fish to further reduce the risk.

Regions of the world that have become more heavily industrialized or mined are also areas that have higher methylmercury concentrations in fish. However, even regions that are considered remote may have methylmercury levels in fish that would be considered above the safe range (Morel et al. 1998). These authors and others have shown that these remote areas of the world are not contaminated by local pollution but rather by mercury pollution that has been transported from around the world through the atmosphere.

*Formation and Transport (Mercury Cycle)*

Methylmercury is a naturally occurring form of mercury that is produced when elemental mercury is biotransformed by bacteria in a body of water. Mercury contamination of aquatic environments occurs naturally as well as through human action. Natural processes which release elemental mercury include volcanic eruptions, forest fires, and rock erosion, although this accounts for only about 10% of all mercury released annually. Anthropogenic sources of mercury include gold mining, burning of fossil fuels
Figure 1A. Fish consumption guidelines by species. Used with permission of Dr. Charles R. Santerre, Purdue University.
(coal burning power plants), cement manufacturing, metal ore smelting (zinc and lead),
and the production of poly vinyl-chloride (PVC) (UNEP, 2013).

Methylmercury is formed when mercury pollutants (either natural or
anthropogenic) are methylated by sulfate reducing bacteria in anoxic surface waters
(Morel et al., 1998). While the exact methods of biological and chemical formation are
poorly understood, it is known that demethylation can occur both biologically and
photochemically (Morel et al., 1998). Once ingested, methylmercury has a half-life of
approximately 44 days. It is biotransformed into inorganic mercury which is then
excreted in the feces (Abelsohn et al., 2002). These authors also stated that
contamination begins at the lowest trophic levels and concentrations increase as one
moves further up the food chain.

**Bioaccumulation and Biomagnification**

The primary source of human exposure to methylmercury is through the
consumption of contaminated fish (UNEP, 2013). Other sources include certain
occupational exposures and the consumption of contaminated foods other than fish. Food
fish become contaminated with methylmercury through consumption of contaminated
prey, and the concentration increases in the fish due to the phenomenon of
bioaccumulation. A related phenomenon is called biomagnification; here, contamination
begins at the bottom of the food web with quite low concentrations of methylmercury
which then increase as one moves up the trophic levels (UNEP, 2013).

The elemental mercury settles on the floor of the water body where it is
biotransformed into methylmercury. It is spread throughout the water column when the
silt and mud on the floor is disturbed. It can then contaminate not only the water but algae within the water. Many of the smallest algal predators are filter feeders like *D. pulex*, which feed on the contaminated algae. As the amount and size of prey consumed increases so does the concentrations of methylmercury contained within the tissue of the next higher predator. The larger the fish the more likely it is to contain a level that may be harmful to humans if more than the recommended serving size of fish is consumed (Boening, 1999). Figure 1B illustrates natural process involved in biomagnification.

**Human Health Effects**

The most important and best studied human health effects resulting from methylmercury exposure are neurological in nature (Mergler *et al.*, 2007). Health problems can occur in children born to mothers who consumed food products that contain methylmercury during pregnancy. Prenatal exposure to methylmercury may occur even if the woman does not consume any methylmercury containing food products during pregnancy due to the long half-life of methylmercury in her body (Adelsohn *et al.*, 2002). Due to the teratogenicity of methylmercury along with the lower required dose for negative health effects in a developing fetus, health problems are often seen in children even when the mother does not show any signs of methylmercury poisoning herself (Mergler *et al.*, 2007; Mozaffarian and Rim, 2006).

Known health effects due to prenatal exposure include the increased likelihood of characteristics of Attention Deficit Hyperactivity Disorder (ADHD), neurodevelopmental delays (ranging from slight delays to mental retardation depending on exposure level), abnormal reflexes and other muscle movement disorders including hyperkinesias,
Figure 1B. Natural processes of biomagnification of methylmercury. Methylmercury bioaccumulates in smaller species which are typical prey species for small fish and biomagnifies up the food chain, leading to the potential for human exposure.
problems sucking/swallowing, impairments of speech (dysarthria) and gait, cerebellar ataxia (Adelsohn et al., 2002; Boucher et al., 2012; Mergler et al., 2007). Other less common outcomes associated with prenatal methylmercury exposure include deformity of the limbs, strabismus, and hypersalivation (Harada, 1995).

While a developing fetus or child is most likely to illustrate the negative health outcomes related to methylmercury exposure, there are other negative health effects associated with post-natal exposures. Post-natal exposures would be those associated with the direct consumption of methylmercury contaminated foods. Older children and adults who have been exposed to a harmful amount of methylmercury may exhibit symptoms such as disturbances with distal sensory, vision field, and auditory perception, ataxia, and tremors (Adelsohn et al., 2002).

Incidence of Human Exposure

An outbreak of methylmercury poisoning occurred in Iraq during the winter of 1971-1972 (Al-Mufti et al., 1976). Barley and grain treated with an organomercury fungicide was distributed for planting (Al-Mufti et al., 1976; Copplestone and Skerfvingi, 1976). However, a portion of this grain was accidentally ground into flour and used to bake homemade breads, while some of the barley was used to feed livestock. Unsuspecting people consumed these baked products as well as the meat from the contaminated livestock. The consumption of these contaminated food products resulted in 6,148 patients being admitted to the hospital after being diagnosed as suffering from methylmercury poisoning. It is suspected that more cases were present but went undiagnosed and untreated due to the rural areas that were affected not having easy
access to medical facilities. Among the 6,148 patients that were admitted to hospitals, 452 died while in the hospital (Copplestone and Skerfvingi, 1976). The number of deaths that occurred is also suspected to be an underestimate due to the fact that many were severely ill when they left the hospitals prior to the completion of treatment, as well as those who never made it to a medical facility. Other cases of methylmercury poisoning occurred in Minamata and Niigata, Japan.

A study in 1995-1996 in the Seychelles looked at the relationship between maternal exposure to methylmercury and neurodevelopmental issues in children. Hair samples were taken from women during pregnancy so that levels present in the hair samples would be more consistent with exposure that occurred during the pregnancy (Crump et al., 2000). Hair samples were also taken from these children at 66 months to determine postnatal exposure levels once the children had begun a fish diet. This study did not find a significant correlation between concentration of mercury in hair samples of the mother and IQ deficits in children at 9 years of age (Alexrad et al., 2007).

Another study was conducted in the Faroe Islands (1986-87) which focused on mercury concentrations in cord blood collected at the birth of the children and IQ deficits at age 7 (Grandjean et al., 1999). Cord blood samples were taken at the time of parturition along with a maternal hair sample. In addition to these samples, hair samples were taken from the children at 1 year and 7 years of age as well as a blood sample at the age of 7 years. Cord blood would only show the prenatal exposure during the 3rd trimester due to the 44-45 day half-life of methylmercury in the body system. Grandjean et al. (1999) found a significant correlation between cord blood mercury levels and developmental delays in children at 7 years. A follow up of this study took place in 2004
(Murata et al., 2004) where the same children which had been tracked through age 7 had hair samples and cognitive tests given at age 14. This same group also reported that there may be long lasting effects from the in utero exposure of methylmercury. One unique source of increase methylmercury exposure among mothers in the Faroe Islands is the consumption of pilot whale meat (Grandjean et al., 1999).

Another study on those living in the Arctic region of Québec, Canada and looked at behavioral outcomes of prenatal exposure to methylmercury in Inuit children (Boucher et al., 2012). Cord blood samples were taken from children born between 1993 and 1998 with 294 mothers and children participating. The children were given neurocognitive tests and mothers were interviewed to gain information of potential confounders. It was found that higher than threshold cord blood mercury concentrations were associated with higher rates of attention type Attention Deficit Hyperactivity Disorder, as reported by the teachers of the children (Boucher et al., 2012). In addition to this study in Canada, a study in China showed that as blood concentrations of mercury increased in children aged 6-10, the incidence of ADHD also increased. (Kargas et al., 2012).

Discussion

*Daphnia pulex* is a species that has been used in research of environmental toxicants for many years and is accepted as a toxicological model by the National Institutes of Health, US Environmental Protection Agency (USEPA), and Organization for Economic Co-operation and Development (OECD). The recent mapping of the *D. pulex* genome makes it a strong model to investigate the effects environmental contaminants may have on gene expression. Since predator induced defenses including
life history and morphological have been linked to the upregulation of genes such as $\text{HOX3}$, $\text{Juvenile Hormone Acid Methyltransferase (JHAMT)}$, $\text{Extradenticle}$, $\text{Escargot}$, and $\text{Methoprene-tolerant (Met)}$, therefore alterations in predator induced modifications on gene expression may be useful to monitor impacts of methylmercury on gene expression (Miyakawa et al., 2010).
LIFE HISTORY

Introduction

*Daphnia* are capable of adjusting different aspects of life in order to ensure the survival of the species. *Daphnia* adjust size, age at sexual maturity, and number of offspring produced in accordance with the particular type of predator that has been detected in the water at that time (Spitze, 1992). The different types of predators that consume zooplankton hunt for food in different ways and this has allowed *Daphnia* to adjust these traits to become more difficult to catch and consume. If the predator that is detected is one that selects for larger individuals as prey items then the *Daphnia* will adjust to this and produce smaller offspring. If the predator detected is one that selects for smaller individuals then larger offspring will be produced (Weber and Declerck, 1997).

*Life History Modifications*

Life history modifications include changes in number of offspring produced, size of offspring produced, and the age at sexual maturation of these offspring (Dodson, 1989). These modifications are dependent on the particular predator that has been detected. In the presence of fish (such as bluegill and zebra fish) kairomone, smaller offspring that reach sexual maturity earlier in life are produced (Dodson, 1989; Leucke *et al.*, 1990). In addition to these differences, broods born under predatory conditions are larger in number. These smaller, younger daphnids also produced offspring that are
smaller in size and reach sexual maturity at a younger age (Dodson, 1989). However, when Chaoborus (phantom midge larvae) predation is detected, offspring are larger and maturity is delayed (Taylor and Gabriel, 1992). These differences are observed over multiple generations and are likely due to the reduction in the number of smaller adults due to predation of smaller daphnids.

Chaoborus predation. When a predator is present in the water system, Daphnia possess the ability to determine the type of predator and undergo changes accordingly. Chaoborus americanus (phantom midge larvae) is an invertebrate species that preys on smaller zooplankton, including Daphnia pulex (Spitze, 1992). This author determined that this leads to the female D. pulex producing offspring that will reach sexual maturity at a significantly later age. A study by Trollian (1995) showed that time to first brood increased by 7.5% in the presence of Chaoborus kairomone compared to a non-kairomone control. It is also known that offspring born to mothers that have been raised in the presence of Chaoborus kairomone also born larger than non-kairomone controls (Trollian, 1995). These size differences continued over all instars which amplified the difference in size between the adult kairomone exposed and unexposed daphnids (Trollian, 1995).

Fish predation. In a natural body of water, a variety of predatory fish that may be feeding on zooplankton, including D. pulex, for example bluegill (Lepomis machrochirius), crappie (Pomoxis nigromaculatus and Pomoxis annularis), and zebrafish (Danio rerio) (Leucke et al., 1990). Fish kairomones have the ability to trigger an
increase in the number of offspring produced, a decrease in the size of offspring produced, and a younger age at first reproduction (Sakwinska, 2000). Life span is not always affected by the exposure to kairomone potentially due to the reduction of triglyceride (TAG) investment in reproduction (Stibor and Navarra, 2000).

*Notonecta predation.* Another invertebrate planktivore is the genus *Notonecta,* which consists of backswimmer aquatic insects. These predators also exhibit size selective feeding habits (Dodson and Havel, 1988). Responses to backswimmer kairomone is similar to the response to fish kairomone in that the result is a larger number of smaller offspring are produced with little effect on the life span of the parent possibly due to the lowered TAG allocation for reproduction (Dodson and Havel, 1988; Murdoch and Scott, 1984).

**Materials and Methods**

**Chemicals**

All chemicals were of analytical grade and were purchased from Fisher Scientific (Waltham, MA), Sigma Aldrich (St. Louis, MO), or Acros Organics (Fair Lawn, NJ) unless otherwise noted. Biotin and manganese(II) chloride tetrahydrate which were purchased from MP Biomedicals (Schaumberg, IL).

*Danio rerio*

*Danio rerio* (zebra fish) were obtained from and housed at the Aquatic Animal Research Core at University of Alabama at Birmingham. The fish being used are
approximately 1 year of age at the time of the experiments. While in use, between two and four *D. rerio* were placed in a 1 L wide mouth glass jar for 24 hours and given a feed rate of ten, four day old *D. pulex* per fish. During this 24 hour period fish are not fed other food to reduce the chance of cross contamination through food source. Fish were kept in a temperature controlled incubator room at 28°C.

While not in use, fish were housed in a 1.8 L plastic tank on the water filtration system, standard for the Aquatic Animal Research Core. Flow rates were kept at levels that would allow full water exchange at least twice per hour within each tank on the recirculation system (Aquaneering, Inc.™). Water used in the system consists of municipal tap water that has been filtered through 5 µm sediment filter (Smith et al., 2013). After passing through the sediment filter, water is then filtered through a charcoal, reverse osmosis, and cation/anion exchange resin manufactured by Kent Marine™. The final step in water preparation, as per protocol in Smith *et al.* 2013, is the addition of synthetic sea salts (Instant Ocean™) to achieve appropriate conductivity (1500 µS/cm). System water pH is maintained by the addition of sodium bicarbonate. While not in use, *D. rerio* are fed a dry food only diet (F08) described as “mixed” diet in Smith *et al.*, 2013. The dry food diet is used to reduce the odds of introducing species, such as rotifers, that would compete with daphnids for food resources. This would result in the possibility of food stress leading to a change in offspring production. Fish were used in accordance with UAB Institutional Animal Care and Use Committee (IACUC).
Daphnia pulex

Daphnia pulex culture was established with clones obtained from Dr. Joe Shaw at Indiana University using conditioned water (COMBO), as described in Kilham et al. (1998). Conditioned water is composed of ultra-pure water (Millipore™ Milli-Q™) with 1 mL/L magnesium sulfate septahydrous, sodium bicarbonate, sodium metasilicate nihydrous, boric acid, potassium chloride, and calcium chloride dihydrous stock solutions as described in Kilham et al., 1998. Media changes were performed every 48 hours with conditioned water and 1 mL/L selenium and animate stocks (Kilham et al., 1998). Animals were housed at a constant temperature of 22ºC with a day/night cycle of 12 hours light and 12 hours dark. Daphnia were fed 80,000 cells/mL of green algae every 48 hours. Green algae, Ankistrodesmus falcatus, was cultured in the labratory of Dr. Julia Gohlke, University of Alabama, Birmingham, using a protocol described in Kilham et al., 1998.

Exposure Paradigms

Fecundity and life span. To assess any effect on life history parameters in response to predator kairomone, daphnids were taken from the 14th brood born to a parent set of 20 females. Non-kairomone controls were taken from a parent set that had been reared in non-fish conditioned water while the kairomone exposed were obtained from a parent set that had been reared in fish conditioned water. Daphnia were reared in individual 50 mL plastic centrifuge tubes, each containing 30 mL of conditioned water (fish conditioned or non-fish conditioned) and the feed rate of Ankistrodesmus falcatus as described
previously under the husbandry section. All daphnids were reared at 22°C with a 12/12 hour light/dark cycle daily.

Media changes were performed every 48 hours to ensure healthy and consistent concentrations of dissolved oxygen and food were available at all times. Media changes were performed by filtering media through a 100 µm nylon mesh cell strainer (Fisherbrand®) with all daphnids being removed via transfer pipet before the tube was refilled and rinsed again to ensure all offspring were counted. Adult female daphnid was placed in the appropriate newly prepared media.

*Effects of methylmercury on life history.* Two experimental groups were used for this study with the first group being started from the 12th brood of 4th generation of daphnids being reared at a constant 22°C. The total offspring produced (n=89) in the same 48 hour period were split into two groups, 44 non-kairomone conditioned water and 45 kairomone conditioned water. The conditioned water stocks were prepared as previously described and were also reared in the same 12/12 hour light/dark cycle constant temperature chamber as previously described. Media changes were performed every 48 hours using a 100 µm nylon mesh cell strainer (Fisherbrand®) with the adult being placed into the appropriate newly prepared media via transfer pipet.

On day 28 of life kairomone and non-kairomone groups were split into 3 groups each, no exposure, methanol, and MeHg. Three 500 mL bottles were prepared with 240 mL kairomone conditioned and 3 non-kairomone media solutions. The specific bottle used was a square shaped glass bottle with a tightly fitting lid. This ensured that in the event the bottle was tipped over it would not spill resulting in a hazardous spill. The
size of the bottle was selected to allow for proper oxygen exchange over the surface of the media to ensure oxygen deprivation would not become an issue. No algae was added to these solutions. The addition of algae to the solutions would add an additional route of exposure to be made available, thus making it impossible to be sure if the effect was from water concentrations or food concentrations. To achieve a concentration of 1600 ng/L of MeHg from the stock solution, concentration 11.4 mg/L, the following calculation was used to gain the volume needed:

\[
(1.6 \times 10^{-3} \text{ mg}) \times (240 \text{ mL}) = 11.4 \text{ mg/L} \times V = 33.68 \mu\text{L MeHg}
\]

A methanol stock solution was then prepared so that 33.68 \( \mu\text{L} \) (0.21 \( \mu\text{g/L} \)) of stock solution was equivalent to the concentration contained within the MeHg stock solution to ensure equal exposure to the methanol vehicle. MeHg in methanol, or methanol alone, was then added to media that had or had not been kairomone exposed; this resulted in four different treatment groups. Daphnids were filtered and offspring harvested and counted. Adult daphnids were then placed into small plastic weigh dishes for holding until all groups were prepared for exposure. Daphnids were carefully added to their respective exposure bottles to avoid physical injury that might confound survival analysis; care was taken to ensure that daphnids were not floating on the surface (due to surface tension) but were swimming in the media. Lids were placed tightly on the jars to prevent any spillage in the event the bottles were disturbed. Bottles were placed back into the 22ºC chamber and the exact time noted.

After exactly 24 hours, bottles were filtered using 100 \( \mu\text{m} \) cell strainer then rinsed and again filtered to ensure that all *Daphnia* are removed from the bottle. Daphnids are washed three times while remaining in the cell strainer by holding the strainer in cups of
clean conditioned water for approximately 1 minute each before the daphnids were placed into a plastic weigh dish for temporary holding until all bottles were complete. The number of live and dead adults was recorded along with the number of offspring present. After all exposures had ended and all daphnids had been washed, daphnids were placed into the respective solutions (kairomone conditioned or non-kairomone) in the tubes prepared earlier and placed back into the 22°C chamber. The number of offspring was counted at subsequent 48 hour media changes with any deaths being noted.

Data was analyzed using JMP® software (JMP® Pro, Version 10. SAS Institute Inc., Cary, NC, 1989-2007). A comparison of brood size was completed using a Wilcoxon Test for both daphnids exposed at 18 and 30 days; this was performed to check for effects at each time point. A post hoc Tukey’s Test was also performed to confirm which groups were significantly different. Life span was also examined over the same exposure groups to gain information regarding any reduction in life span related to exposure to either methylmercury or vehicle control.

*Life span and average brood size in kairomone versus non-kairomone.* To look at differences in kairomone and non-kairomone treated *D. pulex* in typical laboratory settings of 22°C, daphnids were fed a solution containing 80,000 cells/mL of *Anikistrodesmus falcatus*, which was changed at 48 hour intervals. Neonates were taken from same parent sets as in the above experiment and placed into the respective media type (kairomone treated or non-kairomone). For each group, 20 daphnids were randomly selected and placed into 50 mL plastic tubes with 30 mL of media in each tube as seen in previous experiment. No exposures were used and there was no starvation period.
Data was analyzed using JMP® software (JMP® Pro, Version 10. SAS Institute Inc., Cary, NC, 1989-2007). Average life span was analyzed using a Wilcoxon test, and a t-test was used to analyze average brood size.

Results

Life History

Life history kairomone versus non-kairomone. It is important to know the effects that exposure to fish kairomone (D. rerio) have on the life history of D. pulex as this provides a starting point for comparison to study the effects of other treatments. No significant difference (p 0.6723) was found between the life span of kairomone treated and non-

![Figure 2A. Life span of daphnids in non-kairomone and kairomone treated media.](image)

Figure 2A. Life span of daphnids in non-kairomone and kairomone treated media.
kairomone daphnids. One daphnid from the non-kairomone group was removed from analysis due to accidental death. Half of the daphnids remained living at 60 days in non-kairomone media and 58 days in kairomone treated media, respectively. Life span of the longest lived daphnid was also not significantly different, 84 days for non-kairomone and 82 days for kairomone. Life span can be seen in Figure 2A.

**Brood size in Daphnia with and without kairomone.** Brood size was also observed over the lifetime. Average brood size was calculated for each daphnid in the experiment and these averages were compared. A significant difference was found between kairomone and control (p <0.0001), as seen in figure 2B. It is typical for daphnids to reproduce in larger numbers in the presence of fish kairomone, this increases the odds that some of the offspring will survive to reproductive age.

![Figure 2B. Average brood size kairomone vs non-kairomone treatment. Error bars show standard error.](image)
Life span with and without kairomone and chemical exposure. With life history effects known without additional chemical exposures, it was important to know how exposure to methylmercury later in life affected life history traits.

Life span is one life history trait that was observed with daphnids that were exposed to methanol or methylmercury at 30 days of age. There was not a significant difference in the average maximum life span between the groups (p = 0.1380). Figure 2C shows averages of the groups. All daphnids in the methanol control group died (with the

Figure 2C. Average maximum life span across groups exposed at day 30. Methanol Control group was left out of analysis due to only 1 daphnid surviving exposure. Differently labeled groups are significantly different, lettering overlaps show groups in which standard error had overlap. Error bars show standard error.
exception of 1 daphnid) during exposure during this experiment, likely due to handling artefacts, therefore, this group has been eliminated from the analysis. Daphnids in this group were observed to be floating once placed into the media containing the methanol. Any daphnid that died prior to or during exposure was excluded from the analysis.

Life history effects were also tracked when daphnids were exposed to methanol or methylmercury at day 18 of life. There was a significant difference (p <0.0001) across groups. To test if the low number of daphnids that survived more than 2 days post exposure in kairomone and methylmercury group was causing the significance it was removed and reanalyzed, however the results remained significant (p 0.0013). Since

![Figure 2D](image-url)

**Figure 2D.** Average maximum life span across groups exposed at day 18. Differently labeled groups are significantly different, lettering overlaps show groups in which standard error had overlap. Error bars show standard error.
results were unchanged the group was left in the final analysis that is seen in figure 2D. Any daphnid that died prior to exposure was excluded from the analysis to control for early life death as a potential confounder. Results from the day 18 exposure would seem to indicate that there was a negative impact on life span. However results from the day 30 exposure do not show this same significant effect. It is likely that the low number of survivors in the methylmercury exposed kairomone group had a profound effect on this finding.

When datasets from both day 18 and day 30 exposures were combined (to achieve larger sample size) the effect of methylmercury exposure was significantly different from daphnids without methylmercury exposure in the presence of fish kairomone, as seen in Figure 2E (p <0.0001). Methylmercury exposure in the presence of fish kairomone resembled MeHg exposure in the absence of kairomone, which was different from life span of daphnids with or without kairomone in the absence of methylmercury. While a difference was found against the daphnids in the absence of kairomone, the difference was not significant.

Brood size. Brood size is a very important predatory response in Daphnia pulex. An increase in the number of offspring produced in each brood increases the chances that at least some offspring will survive to reach sexual maturity and reproduce, helping maintain the population. If this is diminished, there would be a potential for the daphnids to be eliminated from the body of water.
Brood size was analyzed for each of the two groups mentioned above (18 and 30 day exposures) to inquire if exposure to MeHg or methanol had an effect on brood size. First, all the data was combined into one dataset as time-since-exposure did not have a significant effect on brood size (p 0.4608, data not shown). Figure 2F shows that there is a difference, though not significant, in brood size between the groups with and without kairomone treatment after the starvation period. This is in contrast to what was seen previously with no starvation period, though the reason is not certain.
Figure 2F. Average brood size across combined groups. Data was combined to get overall effect from exposure. Differently labeled groups are significantly different, lettering overlaps show groups in which standard error had overlap. Error bars show standard error.

Brood size was also analyzed for the individual exposure days. Exposure at day 18 was analyzed and is seen in Figure 2G. It was found that two distinct groups were formed along with two intermediate groups (p  0.0081). There was a significant difference between exposure groups with and without kairomone with the exception of the methylmercury exposed kairomone group. Based on data found in this test, there was an increase in brood size when the MeHg exposure group without kairomone when compared to other groups without kairomone, however the increase was not significant.
However, the increase in this group also meant that this group was no longer significantly different from the kairomone control group.

Figure 2G. Average brood size across groups exposed at day 18. Differently labeled groups are significantly different, lettering overlaps show groups in which standard error had overlap. Error bars show standard error.

Brood size for day 30 exposure is seen in Figure 2H. Distinct groupings were found \( p < 0.0001 \), however, small sample size may have skewed some of the data groups. As described previously in the life span analysis, the methanol control group had only 1 daphnid remaining after exposure. In addition to this, that daphnid only reproduced five times after exposure. Another problem group for analysis is found with the control group. While there were survivors in the group, very few of them ever reproduced again \( n=2 \). If these two groups are eliminated the significance is still present \( p 0.0025 \).
Figure 2H. Average brood size across groups exposed at day 30. Differently labeled groups are significantly different, lettering overlaps show groups in which standard error had overlap. Error bars show standard error.

Discussion

Life history modifications are an important response to a predatory environment for *D. pulex*. An increase in brood size in the presence of fish kairomone would increase the chances that at least some of the offspring will survive to a reproductive age. However, there is typically a tradeoff of shorter life span for increased reproduction that is not readily seen in *D. pulex*. This is potentially due to the reduction in triglyceride output for each egg released into the brood chamber (Stibor and Navarra, 2000).
Life Span

This study found that no significant change in life span occurred in the presence of predator kairomone alone, which is consistent with the current literature (Stibor and Navarra, 2000). However, when daphnids were exposed to methylmercury, life span was significantly lessened. Sample size was a limitation in the study due to low survival that may have been due to handling that lead to daphnids floating (surface tension) rather than residing in the water column. However, the data gathered here do suggest that a smaller number of broods may be produced by each daphnid once exposed to methylmercury due to the shortened life span. This means that there would potentially be a decrease in population concentrations due to the exposure to methylmercury.

This reduction in life span may be based on the association between increase brood size in the presence of fish kairomone and the reduction in triglyceride levels that are used for the production of each egg (Stibor and Navarra, 2000 and Sakwinsak, 2000). Methylmercury could reduce gene upregulation associated with this phenomenon resulting in an adult that cannot reduce the triglyceride levels allocated for each offspring and may lead to an early death due to inadequate energy reserves. A reduction in life span would result in a reduction of offspring produced over the lifetime of the animal. This would impact the availability of prey for organisms higher up in trophic level.

Brood Size

An increase in brood size was found in daphnids in the presence of kairomone (8.6/brood vs 3.8/brood, p<0.0001) without any additional chemical exposure. This is similar to what was expected from previous research (Dodson and Havel, 1988; Murdoch
and Scott, 1984). Once methylmercury exposure occurred, there was no significant
decline in brood size in the presence or absence of fish kairomone (p 0.4603). Again,
this experiment may have been limited by the low survival mentioned in the life span
analysis. With the data gathered in this experiment, there would be no significant impact
on the ability of the daphnid residing in a predatory environment to increase number of
offspring produced per brood, which would not directly impact the likelihood of
surviving to reproductive age.

Since there is no decrease in brood size associated with methylmercury exposure,
the availability of *Daphnia* as a food source would be abundant. This would lead to the
availability of more daphnids as prey for predators. Methylmercury concentration
biomagnifies as one moves up the food chain, the increase in brood size associated with
the presence of fish kairomone would result in higher quantities of contaminated
daphnids to be consumed by fish. This could cause increased methylmercury levels in
the tissues of these fish as well as higher trophic level fish. If levels of methylmercury
rise to dangerous levels in the tissue of food fish it could lead to health effects in humans.

When brood size and life span are looked at together, this data implies that while
more neonates are produced this occurs only for a short time after exposure occurs before
the adult dies, possibly from inadequate energy reserves. This would mean that any
offspring produced would have higher levels of methylmercury passed from its mother.
Therefore, when these daphnids are consumed by a prey fish the fish is consuming high
levels of methylmercury and the impacts would be implicated up the food chain and
eventually could have an impact on human health.
MORPHOLOGY

Introduction

A common characteristic of living things is their ability to respond to changes in their environments. In the animal kingdom these responses may be learned or innate in acquisition, behavioral or morphological in manifestation, but in a general sense all of these responses serve to increase the individual’s chances of survival. Responses to avoid predation are key for survival in animals, perhaps with the exception of those at the very top of the food pyramid. The aquatic environment is a complex, interrelated world with multiple trophic levels, varying from algae at the bottom, moving on to zooplankton at the lowest animal level, progressing through various levels of fish and continuing on to large top predators such as gar (Water on the Web, 2004). Daphnia, water fleas, are relatively low in the aquatic food pyramid. As such they are commonly consumed by insect larvae and small fish. It is well established that Daphnia respond to their environment in multiple ways to avoid predation. We will examine in detail several common morphological and behavioral responses to predator presence, and then investigate specific behaviors in *Daphnia pulex* in the presence of *Danio rerio* (zebra fish).

*Morphological Responses*

Several natural daphnid predators have been studied in detail for their ability to induce morphological responses in *Daphnia*. The range of possible morphological changes a daphnid can undergo is amazing, but interestingly there is a specificity in the
daphnid response based on the particular predator that is detected. *Chaoborus* sp. such as phantom midge larvae (*Chaoborus americanus*), small fish species including zebra fish (*Danio rerio*) and bluegill (*Lepomis macrochirus*), and *Notonecta* sp. (backswimmer insects such as *Notonecta hoffmani*) are the three major groups of predators that induce morphological, life history, and/or behavioral responses in *D. pulex* demonstrating a specificity in the morphological response that is based on the nature of the predator, and presumably optimized to maximize survivability in the presence of that specific predator (Kreuger and Dodson, 1981; Dodson 1989; Hanazato *et al.*, 2001). However, each of these predator groups induce different responses from the *D. pulex*. The morphological responses characteristic in *D. pulex* are described in Table 3A.

**Table 3A.**

*Induced Morphological Responses in Daphnia pulex*

<table>
<thead>
<tr>
<th>Predator</th>
<th>Induced Morphological Trait</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Chaoborus</em> spp.</td>
<td>Neckteeth (Krueger and Dodson 1981, Tollrian 1995)</td>
</tr>
<tr>
<td></td>
<td>Fornices (Spitze and Sadler, 1996)</td>
</tr>
<tr>
<td></td>
<td>Body Width Modification (Spitze and Sadler, 1996)</td>
</tr>
<tr>
<td></td>
<td>Body Depth Modification (Tollrian, 1995)</td>
</tr>
<tr>
<td></td>
<td>Tail Spine (Krueger and Dodson, 1981, Spitze and Sadler, 1996)</td>
</tr>
<tr>
<td>fish spp.</td>
<td>Tail Spine (Dodson, 1989)</td>
</tr>
<tr>
<td><em>Notonecta</em> spp.</td>
<td>Tail Spine (Dodson, 1989)</td>
</tr>
</tbody>
</table>

In the presence of *Chaoborus americanus* kairomone, adult *D. pulex* will produce offspring that have one of several morphological changes. Kreuger and Dodson (1981)
found that kairomone-exposed adult *D. pulex* produced two forms of offspring, *D. pulex* and *D. minnehaha*. The two forms of offspring are referred to differently due to the phenotypic changes that must take place to allow the neck teeth to form. Neck teeth are small protrusions which develop from the back of the neck of a kairomone-exposed small (neonate-juvenile) daphnid. The number and size of the neck teeth that an individual daphnid develops is dependent on both the concentration of the kairomone in the water and the amount of biologically available calcium in the water (Ashforth and Yan, 2008). If an insufficient concentration of calcium is present in the water the daphnid may not have the ability to create the extra exoskeleton surface area required for the neck teeth to form.

Exposure to *Chaoborus* kairomone also induces the development of enlarged fornices (Spitze and Sadler, 1996). It is thought that the enlarged width of the fornix, or head shield, (Dartnall *et al*., 2009) in daphnids under predator stress increases the overall fitness of the animal, thus increasing the chances it will survive and reproduce (Repka and Walls, 1998).

Spitze and Sadler (1996) also found that, in the presence of *Chaoborus* kairomone, the body of neonatal *D. pulex* become wider. *Chaoborus* preys on small size daphnids, therefore the body becoming wider increases the overall size of the daphnid and making it less likely to be preyed upon by the phantom midge larvae. A related but separate morphological response is body depth modification (Tollrian, 1995). Changes in body depth similarly correspond to an increase in overall body size. This response however also has a cost, a tradeoff for later time to first reproduction (Tollrian, 1995). Tollrian took measurements of body size during the first instar when the first brood of
eggs was evident. Enlarged body depth is also likely to decrease the chances of the
daphnid being killed by the *Chaoborus*.

The final morphological response is the development of an enlarged tail spine. This response can be induced by *Chaoborus* spp., *Lepomis* spp., and *Notonecta* spp. (Krueger and Dodson, 1981, Spitze and Sadler, 1996, Dodson, 1989). The tail spine is measured from the base of the tail to the tip. Tail spine development is thought to increase the odds of pre-contact or post-contact survival (Havel and Dodson, 1984). This and all other morphological changes are illustrated in Figure 3A.

While the presence of predator kairomone triggers these morphological changes in *Daphnia* species, these changes can be further impacted by environmental factors (Hanazato and Dodson, 1995). Temperature plays an important role in the development of helmets in *D. ambigua* (Hanazato, 1991). This study showed that as temperature increased survival rates decreased for daphnids reared in kairomone (*Chaoborus*) conditioned media but not for those reared in control media, demonstrating an alteration in the predator-dependent response as a function of environmental temperature. This study focused on early life outcomes with the highest demand for energy coming with development of large helmet and decreases with the size of the individual daphnid as well as effects on lifespan.

These interactions are not limited only to temperature but also include influences from pesticides and heavy metals (Lass and Spaak, 2003). The reaction of zooplankton species to environmental contaminants such as heavy metals such as cadmium and nickel, pesticides, and insecticides have been demonstrated to increase the vulnerability of
Figure 3A. Predator induced morphological changes in *Daphnia*. i. Normal morphology. ii. Neckteeth. iii. Body size modification. iv. Enlarged tail spine. v. Helmet formation.
zoo plankton species to predation and resulting in a lowered population in the body of water (Lass and Spaak, 2003).

While some environmental contaminants or conditions can inhibit anti-predator responses, other chemicals actually act in ways that may enhance predatory response. Barry (2001) found that the drugs physostigmine and picrotoxin had significant positive interactions between presence of predator kairomone and exposure to the drug. This effect was measured in size and number of neckteeth produced in D. pulex due to the presence of Chaoborus kairomone. This interesting finding further illustrates the varied, environmentally-dependent changes that D. pulex are capable of producing in response to a complex environment containing kairomone and other chemical / physical factors.

Other studies have also looked at the interaction of predator kairomone and exposure to cadmium, copper, and nickel. Barry and Stoopman (2000) found that sublethal exposure to cadmium was capable of inhibiting the development of induced morphological defenses (Barry, 2002). Copper was found both to increase the number of neck teeth which developed in the presence of Chaoborus americanus kairomone as well as effect a slight decrease on length of neck teeth (Hunter and Pyle, 2004). Nickel can lead to an increased number of neck teeth with increasing concentrations, however the effect is generally not found to be statistically significant (Hunter and Pyle, 2004). Hunter and Pyle (2004) found that while it is not typical for lethal concentrations of these metals to be present in natural water bodies, the concentrations could be high enough to impact predator response in D. pulex.
Behavioral Modifications

Behavioral modifications are critical to the survival of *Daphnia*. Changes in diel vertical migration (Dodson, 1989), swarming behavior (Pijanowska and Kowalczewski, 1997), and swim speed (Gerritsen and Strickler, 1977) aid Daphnids in predator avoidance. While the presence of predator kairomone is one reason for these changes it is not the only cause. Other causes include environmental factors such as temperature, light, and food availability (Dodson, 1989).

*Daphnia* have been found to change depth throughout the day as well as different times of the year. This is presumed to be due to the presence of a predator kairomone in the water (Hanazato *et al.*, 2001). The *Daphnia* have evolved to be able to determine what a safer depth would be for the particular predator that has been sensed. Dodson (1988) found that in the presence of a predator kairomone (*Chaoborus, Lepomis, and Notonecta*) daphnids swam at a significantly different depth when compared to a non-predatory water source.

The overall goal of this study is to investigate the impact that environmental exposure to methylmercury will have on predatory induced life history modifications in *D. pulex*. While previous studies have investigated these outcomes related to exposure to other heavy metals such as cadmium and nickel, the effects of methylmercury has not been investigated.
Materials and Methods

**Chemicals**

All chemicals are of analytical grade and were purchased from Fisher Scientific (Waltham, MA), Sigma Aldrich (St. Louis, MO), or Acros Organics (Fair Lawn, NJ) with exception to biotin and manganese(II) chloride tetrahydrate which were purchased from MP Biomedicals (Schaumberg, IL).

**Danio rerio**

*Danio rerio* (zebra fish) were obtained from and housed at the Aquatic Animal Research Core at University of Alabama at Birmingham. The fish being used are approximately 1 year of age at the time of the experiments. While in use, 2-4 *D. rerio* were placed in a 1 L wide mouth glass jar for 24 hours and given a feed rate of 10 four day old *D. pulex* per fish. During this 24 hour period fish are not fed other food to reduce the chance of cross contamination through food source. Fish were kept in a temperature controlled incubator room at 28°C.

While not in use, fish were housed in a 1.8 L plastic tank on the water filtration system. Flow rates were kept at levels that would allow full water exchange at least twice per hour within each tank on the recirculation system (Aquaneering, Inc.™). Water used in the system consists of municipal tap water that has been filtered through 5 µm sediment filter (Smith *et al.*, 2013). After going through the sediment filter, according to Smith *et al.* (2013), water is put through charcoal, reverse osmosis, and a cation/anion exchange resin manufactured by Kent Marine™. The final step in water preparation, continued Smith *et al.* (2013), is the addition of synthetic sea salts (Instant Ocean™) to
achieve appropriate conductivity (1500 \( \mu \text{S/cm} \)). System water pH is maintained by the addition of sodium bicarbonate. While not in use, *D. rerio* are fed a dry food only diet (F08) described as “mixed” diet in Smith *et al.* (2013). The dry food diet is used to reduce the odds of introducing species, such as rotifers, that would compete with daphnids for food resources. This would result in the possibility of food stress leading to a change in offspring production. Fish were used in accordance with UAB Institutional Animal Care and Use Committees.

*Daphnia pulex*

*Daphnia pulex* culture was established with clones obtained from Dr. Joe Shaw at Indiana University using COMBO solution, as described in Kilham *et al.* (1998). COMBO solution is composed of ultra-pure water (Millipore™ Milli-Q™) with 1 mL/L magnesium sulfate heptahydrous, sodium bicarbonate, sodium metasilicate nihydrous, boric acid, potassium chloride, and calcium chloride dihydrous stock solutions. Media changes occurred every 48 hours with COMBO solution and 1 mL/L selenium and animate stocks (Kilham *et al.*, 1998). Animals were housed at a constant temperature of 22.5\(^\circ\)C with a day/night cycle of 16 hours light and 8 hours dark each day. *Daphnia* were fed 80,000 cells/mL of green algae every 48 hours. Green algae, *Ankistrodesmus falcatus*, was cultured in the laboratory of Dr. Julia Gohlke, University of Alabama, Birmingham, using the protocol developed by the source laboratory at Indiana University (Kilham *et al.*, 1998).
Exposure Paradigms

In order to assess if exposure to MeHg resulted in any changes in magnitude of morphological response, daphnids were taken from 4th generation stock that had been reared at 22°C. *Daphnia* were raised in individual 50 mL plastic centrifuge tubes, each containing 30 mL of prepared COMBO solution as previously described. Full media changes were performed every 48 hours. Broods larger than 3, beginning with the 3rd brood, were harvested and counted every 48 hours during regular media changes.

The first experimental group was started from the 12th brood. Total offspring, 89, were divided into kairomone and non-kairomone stocks with 44 offspring put into the non-kairomone COMBO solution and 45 offspring put into kairomone solution. Kairomone and non-kairomone COMBO stocks were prepared as previously described in the husbandry section. Daphnids were raised at 22°C and kept in 50 mL plastic centrifuge tubes containing 30 mL of kairomone or non-kairomone solution with media changes occurred every 48 hours. Exposure methodology is the same as previously described under “Effects of Methylmercury on Life History” in Chapter 2.

Offspring from broods larger than 3 were collected at each regular 48 hour media changes and put into 50 mL plastic centrifuge tubes labeled for their specific exposure group and solution type, see Figure 3B. Neonates were placed in plastic weigh dishes, to reduce bias, and 5 were randomly taken from each weigh dish and photographed using Olympus Q-Color5 camera with Q-Capture™ version 2 (2009) software and a Nikon 1mm stage micrometer at a 10X magnification.
Combo solution
OR
Combo solution used by *Danio rerio* for 24 hours

Raised for ~30 days

Control, methanol, or methylmercury

Measure length of tail spine and body

Offspring harvested

Figure 3B. Methodology for morphological response.
Images were processed and measurements determined using ImageJ (Schneider \textit{et al.}, 2012). Measurements were taken of tail spine length from base to point of tail spine, body length from top of the head just behind the eye to the bottom of the animal, and body width at the widest part of the body as seen in figure 3C.

A second experimental set of daphnids were collected from the 14\textsuperscript{th} brood from the same parents as the above experimental set. The experiment was repeated with exposure occurring at an age of 18 days. The number of daphnids in MeHg exposure groups was slightly higher at 20 compared to the 15 used in the previous experiment. Protocols described above were repeated identically in this experimental set. Measurement data from both experimental sets were combined for analysis to ensure that all exposure groups are represented.

Measurements were analyzed using JMP\textsuperscript{®} software (JMP\textsuperscript{®} Pro, Version 10. SAS Institute Inc., Cary, NC, 1989-2007). Differences between groups was analyzed using a one-way ANOVA and a post hoc Tukey’s Test. Analysis includes only offspring born after exposure date. Data from both exposures (day 18 and 30) were combined to obtain larger sample size.
Figure 3C. Measurements are demonstrated here. i. Body length. ii. Tail spine length. iii. Body width. Full body length was obtained by adding body length and tail spine length.
Results

**Morphological Response**

*Overall body length.* Morphological changes were tracked using images taken of offspring less than 2 days old following experimental procedures previously described. Overall body length consisted of body and tail spine combined. To obtain this length, measurements of each body and tail spine were added together for each neonatal daphnid. Measurements are shown in Figure 3D. Overall body length was compared across the groups resulting in a p-value of 0.8163. This infers that the overall size of offspring does not change when the entire organism is taken into account.

![Graph showing full body length measurements by exposure group](image)

Figure 3D. Full body length measurements by exposure group. Full body consists of both body and tail spine. Differently labeled groups are significantly different, lettering overlaps show groups in which standard error had overlap. Error bars show standard error.
**Body length and tail spine ratios.** While the overall body length, body and tail spine, did not vary between treatment groups, it is important to know if the tail spine had increased in length with kairomone exposure. To determine this, a ratio was calculated between the tail spine length and the body, and is shown in Figure 3E. This ratio was compared across the groups (p <0.0001) resulting in two distinct groups. Kairomone exposed daphnids had a significantly larger tail spine in relation to body length than those without kairomone exposure. A significant effect on the ability of the methylmercury exposed daphnid to exhibit the predator induced morphological response of an enlarged tail spine was found. The resulting daphnid was not statistically different from the non-kairomone

![Figure 3E. Tail spine to body length ratio by exposure groups. Differently labeled groups (A and B) were found to have differences that were statistically significant.](image-url)
treatment groups. This shows that daphnids exposed to methylmercury during early life in a predatory environment are not able to exhibit this kairomone induced morphological changes leaving the daphnid more vulnerable to predation.

To further investigate the difference this result showed against the results from the full body analysis, the length of the body alone was compared across groups, and is shown in Figure 3F. Differences in body length were also statistically different with a p-value of <0.0001. The statistically significant difference between methylmercury exposed daphnids in the kairomone treatment and methanol exposed and un-exposed kairomone treatments shows that there is a significant effect on body length. This difference would infer that the ability of the methylmercury exposed daphnid to exhibit

Figure 3F. Body length by exposure groups. Differently labeled groups (A and B) were found to have differences that were statistically significant. Group labeled AB was not significantly different than either group A or group B.
the reduction in body length related to kairomone exposure is diminished. The methylmercury exposed non-kairomone treatment group shows a difference between each of the groups, however is not significantly different than either group. This means that the methylmercury had an effect on the non-kairomone group though the effect was not significant.

Tail spine length alone was also examined across treatment groups, as shown in Figure 3G. Mean tail spine length was compared across the treatment groups (p <0.0001) with kairomone treatment showing a significant increase in both the kairomone and kairomone plus methanol exposure groups; however, the group with daphnids exposed to

![Figure 3G. Tail spine length by exposure groups. Differently labeled groups (A, AB, and C) were found to have differences that were statistically significant. Group labeled AB was not significantly different than group A.](image)
kairomone and methylmercury is statistically different from all others. In the presence of kairomone, the methylmercury exposed group most closely resembled the non-kairomone groups. The tail spine was slightly larger in the kairomone with methylmercury group; however the difference was not statistically significant. In the absence of kairomone, there was no significant difference among any of the exposure groups. This shows that methylmercury significantly inhibited the ability of the daphnid to exhibit the morphological defense of a lengthened tail spine.

Body width. With the difference in body length, it was of interest if the width of the body also changed between exposure groups. Body width measurements are seen in Figure 3H.

Figure 3H. Body width by exposure groups. Differently labeled groups (A, and B) were found to have differences that were statistically significant. Group AB was not significantly different than group A or B.
The width of the animal was measured at the widest part of the body on each animal. Significant differences were seen between body width at the widest part of the body in kairomone treated and non-kairomone treated with exception to if the daphnid was exposed to methylmercury (p <0.0001). Methylmercury exposure decreased the width, though not significantly, of the non-kairomone treatment group and had incomplete inhibition of the expression of the morphological response in the kairomone treatment group.

**Body Width and Tail Spine Length Ratio**

Both body width and tail spine length were altered in the presence of kairomone and morphological defenses were impacted by the exposure to methylmercury. With the individual changes analyzed (see Figures 3G and 3H), it was of interest if the two morphological changes were proportional. Tail spine to body width ratio is seen in Figure 3I. Three significantly different groups were found with tail spine to body width ratio (p <0.0001). All non-kairomone treatment groups fell into one group, showing no effect from exposure to methylmercury. A significant difference was found between methylmercury exposed kairomone treated daphnids and kairomone treated daphnids in the no exposure and methanol exposure groups. While the predator effect was inhibited in the methylmercury exposed group, exposure did not completely eliminate the effect as the group was significantly different from non-kairomone treated groups.
Figure 3I. Tail spine to body width ratio by exposure groups. Differently labeled groups (A, B and C) were found to have differences that were statistically significant.

Discussion

*Kairomone Induced Morphological Changes*

In a predatory environment, *Daphnia pulex* have the ability to exhibit phenotypic plasticity. Phenotypic plasticity refers to the ability of an animal to change expression of specific genes that induce beneficial changes. These changes include morphological changes of body and tail spine. Other studies have investigated the effect of dissolved metals such as copper (Mirza and Pyle, 2009) and cadmium (Barry, 2002) although none have investigated the impact of the common environmental pollutant methylmercury.

Previous studies have reported that body size is reduced in the presence of fish kairomone (Dodson, 1989). The results presented in this chapter, however, found that
overall body length (body and tail spine) was not significantly different in the presence or absence of kairomone or methylmercury. This infers that it is not necessarily the overall body size that serves as the anti-predator morphological change in *Daphnia pulex*. Since fish are sight hunters, a reduction body size would make the daphnid less likely to be seen by the predator (Spitze and Sadler, 1996; Tollrian, 1995). Body length among the treatment groups was compared resulting in a significant difference between daphnids born in the presence or absence of fish kairomone and the effect of early life exposure to methylmercury. While exposure to fish kairomone significantly decreased the length of the body, the addition of methylmercury resulted in the inhibition of morphological changes in exposed daphnids. Length of tail spine was found to be smaller in the presence of methylmercury as well when compared to kairomone exposed daphnids in the absence of MeHg. If the daphnid is not able to express these morphological adaptations, its chances of surviving until sexual maturity may be diminished. These findings were again confirmed when a ratio was used to compare tail spine length in relation to body length. This shows that as tail spine becomes longer the body becomes shorter, which is confirmed by the insignificant difference in full body length and that early life exposure to methylmercury inhibited protective morphological changes.

Body width was also of interest as a decrease in width would also make it more difficult for a sight predator to see and catch its planktonic prey, increasing the likelihood that the prey organism will survive to reproduce (Spitze and Sadler, 1996; Tollrian, 1995). Fish kairomone exposure resulted in a reduced body width when compared to control daphnids. Methylmercury exposure exhibited incomplete inhibition of the morphological response of body width modification. A ratio of tail spine length and
body width was used to test if morphological changes are consistent over the entire body of *Daphnia pulex*.

Overall, methylmercury had a significant effect on the ability of neonatal *Daphnia pulex* to express phenotypic plasticity in a predatory environment. This reduces the likelihood that the neonate will survive through first reproduction, potentially affecting overall population numbers in a body of water.
CONCLUSIONS AND FUTURE RESEARCH

Conclusions

Mercury is a heavy metal that is readily dissolved in water, and may be released by both anthropogenic and natural sources. It is then transformed into methylmercury by sulfate reducing bacteria found in the water. Methylmercury has a long half-life and bioaccumulates in the body and biomagnifies up trophic levels in aquatic environments. This leads to human exposure through the consumption of contaminated fish meat. If blood concentrations are too high there is a significant risk of mental developmental delays to unborn children as well as mental and physical disabilities in adults. It was found that early life exposure to methylmercury reduced the ability of neonatal *Daphnia pulex* to undergo the morphological responses that the species has evolved to increase chances of survival in a predatory environment. These morphological changes are the result of phenotypic plasticity through the upregulation of specific genes in response to the detection of predator kairomone. Exposure during adulthood resulted in the reduction of brood size in daphnids reared in the presence of fish kairomone. This implies that early life exposure to methylmercury may affect gene expression.

The increase in brood size found in the presence of fish kairomone was not affected by exposure to methylmercury; therefore a higher concentration of daphnids would become available for predation. When this is coupled with the reduced ability of the daphnids to evade predation, it would lead to a higher number of contaminated daphnids being readily available for consumption by prey fish. Any increase in
consumption rates would lead to higher concentrations of methylmercury in the tissue of fish, increasing as biomagnification occurs up the food chain. This would lead to an increase in waterways that are considered unsafe for fish consumption.

It is known that the presence of a predator kairomone causes the upregulation of several genes in *D. pulex* such as *Hox3, Juvenile hormone* acid methyltransferase (*JHAMT*), *Extradenticle, Escargot*, and *Methoprene-tolerant (Met)* (Miyakawa *et al.*, 2010). Exposure to methylmercury showed a decrease in the ability of *D. pulex* to undergo phenotypic changes associated with predator induced morphological changes. Since these gene upregulations are necessary for daphnids to exhibit predatory responses, it could be implied that methylmercury is affecting gene regulation in exposed individuals.

While information regarding fish advisories is readily available online for people to see, not all people have access to internet or possess the knowledge that these reports are available to them. Fish advisories would need to become more easily accessed by those who rely heavily on fishing as a food source for their family. Even with knowledge that consuming fish from a contaminated waterway, some families have few options due to financial hardships. This is a situation that many families worldwide face each day.

While information about the health effects of methylmercury is available, research is typically conducted on a single chemical with no additional stressors. This is not how typical exposures occur. Therefore, it is important to build reliable multi-stressor models to gain knowledge about exposures in a situation more closely related to a real world situation. Families that fish contaminated waterways out of hardship are facing additional stress such as starvation, the possibility of losing their family home or
even their family. It is possible that stressful situations such as this may make an exposed person more susceptible to the negative health effects associated with environmental exposures. Several studies have investigated the interaction between heavy metal exposure and the immune system in various species, however the body of literature is not conclusive (Snoeijs et al., 2004).

Future Research

Sample size was one of the limitations of this study; therefore, this research would benefit from an additional study using larger sample sizes in each of the six exposure groups. A larger sample size would increase the reliability of the results over the entire population. Additionally, this would help to eliminate the issues seen here regarding the elimination of certain exposure groups from analysis due to inadequate data.

With gene upregulation being at the root of predatory responses in daphnids, a study should be conducted that would test for alterations of gene regulation. Several studies have looked into which genes are upregulated in response to a predatory environment in *D. pulex* but no studies have been conducted that test potential impacts related to heavy metal exposure. Such a study is currently underway but results were not yet available. However, additional studies with larger sample sizes for each group would be beneficial. Larger sample size would allow for additional testing to be carried out on offspring born at different time points after exposure. This would increase knowledge of how long methylmercury remains in the body of *Daphnia*.

Additionally, a study should be conducted that would allow the offspring of exposed daphnids to grow into adulthood and life history effects to be analyzed. This
study could also consider potential changes in morphological response in generations born to daphnids with early life exposure. Knowledge may be gained on hereditary changes in gene expression.
LIST OF REFERENCES


APPENDIX A

Megan: Here is a clean copy. You have my permission to include in your thesis. Good luck in your efforts. Best regards, Charlie

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From: Megan L Steed [mailto:mlsteed@uab.edu]
Sent: Friday, February 28, 2014 1:13 PM
To: Santerre, Charles R
Subject: permission to use florida fish card

Dr. Santerre

I was writing to attempt to gain permission to use your fish consumption card in my thesis. I am researching the effects of maternal exposure to methylmercury in *Daphnia pulex* in a new multi stressor model and would love to include the card for my background on safe fish consumption.

Since the thesis will be published I am needing a permission statement in order to use the image.

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