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A Caenorhabditis elegans Model to Detect Developmental Teratogens with Adverse Reproductive and Metabolic Effects

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A Caenorhabditis elegans Model to Detect Developmental Teratogens with Adverse Reproductive and Metabolic Effects

by

Alexis A. Killeen

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medical Sciences with a concentration in Molecular Medicine

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DEDICATION

I would like to dedicate this first, to my parents, Robert and Roberta Killeen, and to my brother, Jonathan. Thank you Mom and Dad for being so incredibly supportive throughout my life, and especially in these past four years. Thank you for the late night calls and the leftovers. I couldn’t have done this without you. Thank you Jonathan, your sense of humor never failed to grant me a new, lighter perspective on any challenge I had.

I would also like to dedicate this to my abuelos, Robert and Concepción Iglesias. Thank you for reminding me to be dogged, but to have balance. To pursue my love of science, but to never stop painting along the way.
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<th>Description</th>
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<tbody>
<tr>
<td>AC</td>
<td>Adenylyl Cyclase</td>
</tr>
<tr>
<td>AhR</td>
<td>Aryl hydrocarbon Receptor</td>
</tr>
<tr>
<td>AOI</td>
<td>Area of Interest</td>
</tr>
<tr>
<td>As(III)O</td>
<td>Arsenic(III)Oxide</td>
</tr>
<tr>
<td>BAP</td>
<td>Benzo-α-pyrene</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown Adipose Tissue</td>
</tr>
<tr>
<td>BLAST</td>
<td>Basic Local Alignment Search Tool</td>
</tr>
<tr>
<td>BPA</td>
<td>Bisphenol-A</td>
</tr>
<tr>
<td>BPS</td>
<td>Bisphenol-S</td>
</tr>
<tr>
<td>C/EBP-a</td>
<td>CCAAT Enhancer Binding Protein-alpha</td>
</tr>
<tr>
<td>CARS</td>
<td>Coherent Anti-Stokes Raman Scattering</td>
</tr>
<tr>
<td>CDC</td>
<td>Centers for Disease Control</td>
</tr>
<tr>
<td>Cd-Cl</td>
<td>Cadmium Chloride</td>
</tr>
<tr>
<td>CHD</td>
<td>Congenital Heart Defects</td>
</tr>
<tr>
<td>CS</td>
<td>Cigarette Smoke</td>
</tr>
<tr>
<td>CSE</td>
<td>Cigarette Smoke Extract</td>
</tr>
<tr>
<td>DES</td>
<td>Diethylstilbestrol</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl Sulfoxide</td>
</tr>
<tr>
<td>DR</td>
<td>Dietary Restriction</td>
</tr>
<tr>
<td>EPA</td>
<td>Environmental Protection Agency</td>
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</table>
FDA .................................................................................. Food and Drug Administration
GD ...................................................................................... Gestational Day
GFP ...................................................................................... Green Fluorescent Protein
HTS ...................................................................................... High Throughput Screen
IPTG ................................................................................. Isopropyl β-D-1-thiogalactopyranoside
JTT ...................................................................................... Jones, Taylor, and Thorton
LPL ...................................................................................... Lipoprotein Lipase
MSA ...................................................................................... Multiple Sequence Alignment
MT1 ...................................................................................... Metallothionein 1
MT2 ...................................................................................... Metallothionein 2
MUSCLE ................................................................. Multiple Sequence Alignment by Log-Expectation
NGM ...................................................................................... Nematode Growth Media
NHR ...................................................................................... Nuclear Hormone Receptor
NNI ...................................................................................... Nearest Neighbor Interchange
PAM ...................................................................................... Point Accepted Mutation
PEPCK ................................................................. Phosphoenol Pyruvate Carboxykinase
PLIN ...................................................................................... Perilipin
PND ...................................................................................... Post-Natal Day
PPARs ............................................................... Peroxisome Proliferator Activated Receptors
PPM ...................................................................................... Pharyngeal Pumps per Minute
RNAi ...................................................................................... RNA interference
ROS ...................................................................................... Reactive Oxygen Species
RXRa ...................................................................................... Retinoid X Receptor alpha
ABSTRACT

Obesity is a current epidemic in the United States. Greater than one third of adults and nearly one fifth of children are classified as obese (Ogden, 2014). Increased awareness of this problem has triggered interest into the potentially causal agents in our environment. These agents, termed obesogens, are a type of teratogen hypothesized to work during embryonic development by programming offspring to store excess fat. A type of teratogen, obesogens are also typically reprotoxic, affecting germline development and embryonic viability. These noxious teratogens span a variety of different chemical classes including estrogen mimics, metals and metalloids, biocides, and even voluntary exposures like cigarette smoke. The goal of this work is to use the soil nematode, Caenorhabditis elegans to model the reprotoxic and obesogenic events that take place as a result of chronic, low-dose exposure to these agents. C. elegans is especially well suited to this end. The organism has a straightforward biology, a short lifespan on the order of days, and relevant lipid metabolic gene homology to humans. We characterize here the resultant effects on egg-laying, hatching, and embryo viability after chronic parental exposure to obesogens. Through this, we are able to best recapitulate the low-dose exposure for our obesogen screen. Here, chronic, sub-lethal parental exposure to the obesogens screened yielded viable offspring that exhibited increased percentages of body area lipid stained and induced differences in stained lipid localization. Reduced pharyngeal pumping rates in two of our offspring groups highlights the potential for lasting changes on behavioral
phenotype as well. Lastly, we employed phylogenetic guided analysis to functionally assess putative C. elegans nuclear hormone receptors (NHRs) to human Peroxisome Proliferator Activated Receptors (PPARs). These human PPARs have implications in lipid metabolism and obesity. We report here novel findings that NHR-85 and SEX-1 may act to repress lipid storage in the nematode, thereby serving as potential homologs of human PPARα. The results presented in this study highlight the lasting effects of chronic parental exposure to noxious environmental teratogens on the function of the reproductive system. Furthermore, the nematode can be used as an in vivo model to screen sublethal doses of these teratogens for their potential to act as obesogens. Bioinformatics guided analysis of C. elegans NHR homologs to human NHRs can continue to yield novel insight on this fat storage and its regulation in the nematode.
CHAPTER ONE:

INTRODUCTION

It is estimated that direct exposure to environmental pollutants accounts for nearly 10% of today’s global disease burden (Briggs, 2003). We can safely assume this estimate may even be higher if we account for diseases resulting from indirect, prenatal exposures. As reviewed by Sigelman and Rider, until the 1940s, the uterus housing an embryo was considered a vessel impervious to deleterious environmental influence. The placenta was viewed as the protective organ, filtering out maternal exposures including pharmaceuticals, cigarette smoke, and alcohol (Sigelman and Rider, 2011). However, later work detailing the persistent effects of teratogens has revealed otherwise. Today, the Fetal Origins of Adult Disease is a related, rapidly growing hypothesis applied within several fields to investigate the developmental causes for adult disease (Barker et al., 2002). In the study of obesity, one research focus investigates potential causal teratogens termed ‘obesogens’. These compounds are examined for their ability to program in utero offspring energy balance and metabolism in such a way that they predispose the organism to excess weight gain.

1.1 Defining a Teratogen

The meaning of the word teratogen is embedded within its Greek roots: *tera-*, translating to ‘monster’, and *-ogen*, translating to ‘the origin of’. Thus, ‘teratogen’ is a word used to describe an in utero exposure that induces disease in the offspring. One of the most reknown examples is the drug thalidomide, which was commonly prescribed
to European women in the 1950’s as a means to ameliorate morning sickness during pregnancy. Subsequently, a large push was made by the William S. Merrell Chemical Company to introduce this drug to the United States (Harris, 2010).

Working for the United States Food and Drug Administration during that time, Dr. Francis Oldham Kelsey refused to authorize the entry of this drug because of the insufficient evidence concerning its effects on the developing fetus (Harris, 2010). Meanwhile, thousands of children in Europe were born with shortened or deformed limbs as a result of prenatal exposure to this drug, which was shown decades later to interfere with proper limb bud development via anti-angiogenic mechanisms (Therapontos et al., 2009). Dr. Oldham Kelsey’s efforts were pivotal in the establishment of stringent guidelines for drug evaluation and approval in the United States, ultimately preventing thousands of American children from incurring a similar fate. She was recognized for these endeavors, receiving the President’s Award for Distinguished Federal Civilian Service in 1962 (Peters and Woolley, 1962; FDA, 2013). Her efforts continue to be recognized and revered in the FDA’s Frances O. Kelsey Award for Excellence and Courage in Protecting Public Health, of which she was the inaugural recipient in 2010 (Harris, 2010; FDA, 2013).

Teratogens however, are not limited in their definition to solely voluntary pharmaceutical exposures. A host of environmental pollutants and recreational exposures have been shown to function as teratogens. Many environmental compounds have been shown to be capable of bioaccumulating in reproductive tissues and crossing the placental barrier. Consequently, they may impede fertilization and embryonic development. In those offspring that do survive to parturition, these agents
may induce disease. To address these deleterious effects of common agents in our environment, this project focuses on four major categories of teratogens: estrogen mimics, biocides, metals and metalloids, and cigarette smoke.

1.2 Teratogenic Estrogen Mimics

1.2.1 Bisphenol-A

Bisphenol-A (BPA) is a synthetic estrogen originally developed in 1891 that was widely implemented in the production of epoxy resins, plastics, and papers during the mid-1950s (Vogel, 2009). The primary source of exposure to BPA for children is through ingestion where they may be consuming as much as 1.19 μg/kg-BW-day, as school lunches are typically prepared from food in cans lined with the chemical (Hartle et al., 2015). For adults, the primary route of exposure is presumed to be through handling of BPA-containing papers such as receipts (Liao and Kannan, 2011). BPA's ubiquity in consumer products is what ultimately led to its discovery as a potent aneuploidy-inducing substance in the mouse oocyte.

In 2003 Patricia Hunt noted that damage to polycarbonate cages and water bottles caused by an acidic detergent in the animal housing facility associated to an elevated incidence of control mouse oocytes displaying an increased rates of aneuploidy. Mice housed in damaged cages were postulated to be more likely to absorb the plasticizer BPA, which could then reach the mouse gonad and disrupt meiosis. The group was later able to experimentally confirm this hypothesis by systematically damaging cages and water bottles under controlled conditions with the same detergent (Hunt et al., 2003). This finding was replicated almost a decade later in the nematode Caenorhabditis elegans and shown to reduce embryonic viability in a
dose-dependent fashion. Specifically, BPA interfered with the proper formation of the synaptonemal complex that forms prior to homologous recombination in developing nematode oocytes and induced chromosome nondisjunction (Allard and Colaiácovo, 2010). Meiotic nondisjunction is a well-known risk factor for the development of birth defects such as trisomy 21.

Additionally, bisphenol-A is also a very potent methyl acceptor, capable of drastically altering the imprinting patterns of genes during the first and crucial early stages of embryonic development. Dolinoy et al. demonstrated with an epigenetic biosensor- the viable yellow Agouti (A\(^{vy}\)) mouse, that maternal BPA administration induced offspring genome hypomethylation as detected by an increased number of yellow coat-colored mice. In this mouse model, the Agouti gene encoding yellow coat color is constitutively active and expression is inversely related to the amount of genome-wide methylation at its loci. The effects were prevented when BPA was administered in conjunction with the methyl donor, folate, resulting in the production of more pseudoagouti brown and 'mottled' (combination pseudoagouti brown mottled with yellow coat color) offspring (Dolinoy et al., 2007). Taken together, these studies highlight the range and potential effects BPA may have on fertility and offspring disease, from meiotic disjunction to aberrant genome-wide epigenetic modification.

1.2.2 Diethylstilbestrol

Diethylstilbestrol (DES) is a second, well-documented teratogenic estrogen mimic that has been shown to induce similar meiotic abnormalities in the germline of C. elegans (Goldstein, 1986). Sir Edward Charles Dodds, an investigator of synthetic estrogens and their activities, including BPA, initially developed DES in 1938 (Dodds,
After the news of its potency was revealed, it was heavily marketed and prescribed from the 1938-1971 as a pharmaceutical to prevent miscarriage by supplementing the mother's natural supply of estrogens (CDC, 2003). In 1952 research revealed that DES had no effect on pregnancy maintenance, but unfortunately the drug was not banned by the FDA until 1971 (Ferguson, 1952; Herbst, 1971; CDC, 2003). Around this time in utero exposure to DES was being linked to development of vaginal and cervical clear cell adenocarcinoma in the resultant daughters, in addition to the formation of T-shaped uteri that commonly rendered these women infertile (Verloop et al., 2010; Rennel, 1979). These findings have been further replicated in rodent models, where prenatal exposure to 67μg/kg of DES on GD (gestational day) 13, a critical period of embryonic reproductive organ development in the mouse, was found to induce a cervical cancer and hypertrophy of the reproductive tract in Balb/C mice offspring aged to 5 months (Zulfahmi et al., 2013). The epidemiological findings, corroborated experimentally, illustrate diethylstilbestrol as a teratogenic agent capable of having lasting, life-altering, and deleterious effects on offspring development.

1.3 Teratogenic Biocides

1.3.1 Tributyltin

Tributyltin (TBT) is an antifouling paint additive developed in the 1960’s to minimize algal and barnacle growth on the hulls of ships (Bray and Langston, 2006). It has since been deemed “the most toxic substance ever deliberately released into the marine environment” (Goldberg et al., 1986; Evans et al., 1995). Human exposure to organotins in general can occur through ingestion of contaminated seafood, which has been estimated to be on average between 2.5 and 3.2 ng/kg/day (Rantakokko et al.,
Early work in the 1980’s revealed this compound to be a potent endocrine disruptor inducing imposex male gonad formation in female mollusks; and ultimately responsible for the collapse of a prominent oyster farm in France (Bray and Langston, 2006). TBT is especially dangerous because of its prolonged half-life, which can range from roughly 2 years in an aerobic environment, to a projected estimate of 3 decades under anaerobic sediments (Dowson, Bubb, and Lester, 1996). Because of its primary use on boats, TBT pollution is most concentrated in regions with major ports (Bray and Langston, 2006).

Because TBT leaches into the water from the coating on boats, research heightened in the late 1980’s and early 1990’s to understand its potential noxious effects on human health. In fact, Ema et al. in 1995 found that prenatal exposure during the period of late organogenesis in the Wistar rat to sub-embryo toxic concentrations of TBT induced a significant increase in the number of offspring born with cleft palate (Ema et al., 1996). One year after the publication of these findings, Japan banned production of TBT-containing paints (Adamson and Brown, 2002). Recently however, research has revealed the potential of TBT to adversely affect methylation patterns in developing oocytes and viability of preimplantation embryos, highlighting the potential for teratogenicity at the earliest stages of embryonic development (Huang et al., 2015).

1.3.2 Triclosan

Triclosan is a second well-known biocide that was commonly used in formulations for detergents, deodorants, soaps, papers and adhesives (EPA, 2010). Only within the past decade has the slow eradication of this constituent from consumer goods begun to take place. However, its implementation in Colgate Total® toothpaste
has persisted and was advertised as recently as 2013 (Household Products Database, 2015; colgatetotal.com). In 2013 the CDC National Biomonitring Program did not view triclosan as teratogenic; even though evidence from a study in 2010 had previously shown that pre- and peri-natal exposure in the Wistar rat to doses of triclosan as high as 50- and as low as 1-mg/maternal-kg/day are enough to significantly delay sexual maturation as indicated by vaginal opening in female offspring (Rodríguez and Sanchez, 2010). Furthermore, this same study revealed that exposure to the highest dose reduced the number of live births and survivability to postnatal day 6 of the resultant offspring (Rodríguez and Sanchez, 2010). Because triclosan has been so widely used up until the past decade, the amount of literature detailing its noxious effects on offspring health is limited. Indeed, the dose tested in the Rodriguez study is well below the level of 0.0092 μg/kg estimated for human consumption (EPA, 2008). Given its plasma half-life of 21 hours and its lipophilic nature, triclosan may potentially bioaccumulate, which would extend its effects beyond the initial acute exposure (Sandborough-Englund et al., 2006). It is important to note that given triclosan’s prior ubiquitous presence in sanitation products, exposure to this agent likely occurred at small doses on a daily basis. This opens up the possibility for lasting ‘low dose effects’ that have been observed with other agents such as BPA (vom Saal et al., 1998). Thus, the scope of triclosan’s teratogenic effects still warrant further investigation in the scope of chronic, low dose exposure.

1.3.3 Organophosphates

A third, and environmentally relevant biocide for the state of Florida, is the organophosphate pesticide Fenthion. Phased-out in all states except Arkansas,
Missourri, and Florida, this pesticide is used to combat mosquitos and dragonfly larvae (EPA, 2001). The half-life in aqueous environments can be as many at 21 days, and in soils as great as 34 days, where absorption can occur through inhalation or skin contact (Wauchope et al., 1992).

Fenthion specifically was initially not regarded as teratogenic (Shephard, 1986). However, in 1995 Cosenza and Bidanset revealed a related organophosphate, chlorpyrifos, was particularly toxic to embryonic rat midbrain neurons in culture (Cosenza and Bidanset, 1992). In 2004, the Slotkin group found that exposure during the later part of the prenatal development in the Sprague-Dawley rat to the organophosphate pesticide chlorpyrifos impaired adenyyl cyclase (AC) mediated signaling in the embryonic brain. Authors highlight that AC signaling is especially important in neurodevelopment by aiding in the coordination of neuronal differentiation, growth, and apoptotic pathways (Meyer et al., 2003). Recently, Fenthion was evaluated in the context of a combination pre- and perinatal exposure model for its histopathological effect on the developing Wistar rat brain. Oral administration via drinking water of 61 mg/kg of maternal body weight through the period of 14 days pre- and post-birth promoted reactive oxygen species (ROS) generation and induced neuronal cell death in the cerebrum and cerebellum of offspring. Additionally, authors noted that higher dosages administered to pregnant rats resulted in embryonic lethality and reduced fecundity (Amara et al., 2014). Fenthion's effects on the developing rat brain are in-line with those of other chemicals of its same class, highlighting the teratogenic potential of this conventional insecticide in a mammalian system.
1.4 Teratogenic Metals and Metalloids

1.4.1 Arsenic

Inorganic arsenic pollution has resulted from past-day application of agricultural arsenic-containing pesticide to crops, and present-day industrial processes of smelting and manufacturing of fabrics and electronics. The most common routine exposure of the public to arsenic trioxide [arsenic(III)oxide], the major constituent of arsenic contamination, is through groundwater and particulate matter carried in the atmosphere (ATSDR, 2007).

The teratogenicity and reproductive toxicity of inorganic arsenic has a documented history spanning multiple decades both in epidemiological and experimental studies (for a thorough review, see Golub and Mactintosh, 1998). For example, a study in the early 1990’s found an increased incidence of spontaneous abortion and stillbirth in mothers drinking arsenic-tainted well water in southeast Hungary when compared to mothers in neighboring regions without the exposure (Börzsönyi et al., 1992). Recently, a prospective birth cohort study as part of the Metals Assessment Targeting Community Health found after adjusting for other heavy metals (lead and manganese) in the circulation of mothers near a North Dakota Superfund site, that inorganic arsenic was most closely associated with reduced birth weight, head circumference, and gestational length (Claus Henn et al., 2016). Experimentally, various forms of inorganic arsenic have been shown in multiple rodent models to induce similar effects on fertility including increased pre- and postnatal mortality, reduced fetal weight, and in one instance, reduced maze-learning performance of resultant offspring (Golub and Mactintosh, 1998).
1.4.2 Cadmium

Cadmium is a heavy metal commonly used in the production of batteries, and to a lesser extent, pigments, paints, and plastics. A common source of environmental pollution however, is airborne exposure from the mining and refinement process for metals like copper and iron. The primary route of cadmium exposure for the general public is through food, however smoking has been shown to greatly increase the levels of circulating cadmium raising blood levels from the population average of 0.38ug/L to levels as high as 3μg/L (ATSDR, 2012).

Few epidemiological studies exist evaluating the teratogenicity of cadmium specifically on offspring, however the metal has been shown to accumulate in the follicular fluid of actively smoking women (Sioen et al., 2013; Zenzes et al., 1995). The evidence of bioaccumulation in this tissue demonstrates its potential to have lasting effects on developing oocytes and embryos, even beyond the initial exposure.

Experimentally, constant exposure in mice to cadmium via osmotic minipump to 1.4, 2.8, or 5.6 μmol Cd over GD5-18 has been shown to significantly increase the number of fetal soft tissue and skeletal abnormalities observed from the two highest dose groups (Mahalik et al., 1995). Distribution of cadmium in maternal, placental, and fetal tissues revealed that over time, cadmium levels in the placenta remained consistently greater than noted in the fetus, and in the fetus cadmium levels decreased over the 6 days of assessment. The authors note that these results indicate tissue- and time-dependent susceptibilities, where the placenta may be more at risk than the embryo to the toxic effects of cadmium later in development (Mahalik et al., 1995). These ideas were supported by a subsequent mouse study in 2012 that found cadmium
was capable of inducing oxidative stress and cell death in the placenta. The effects on the placenta were postulated to reduce nutrient transfer to fetuses, ultimately causing the striking and significant reduction in fetal weight and size (Wang et al., 2012).

1.5 Cigarette Smoke as a Multifaceted Teratogen

Cigarette smoke (CS) is a well-documented teratogen that is actually a heterogeneous mixture of over 4000 noxious compounds. These compounds include heavy metals and metalloids like cadmium, arsenic, and lead. Cadmium as mentioned earlier has been shown to bioaccumulate in the follicular fluid of actively smoking women pursuing assisted reproductive treatment. Interestingly, those exhibiting the highest cadmium concentrations were also the least likely to conceive (Zenzes et al., 1995; Neal et al., 2008). Additionally, the polyaromatic hydrocarbon benzo-α-pyrene, has been detected in the follicular fluid, granulose cells, and early embryos of actively smoking women. In the granulosa cells, the amount of DNA adduct formation was found to increase in a dose-dependent fashion with maternal smoking frequency (Zenzes et al., 1998). The later data highlights the potential for this agent to have similar noxious effects on the germ cells and developing embryo.

In general, CS has been shown to be associated with a variety of offspring birth anomalies and diseases, often in a dose-dependent fashion. A study from Nunavut for example, found that the risk for preterm birth and low birth weight increased by 2- and 6-fold respectively, in mothers from the highest smoking category that consumed at least 10 cigarettes/day (Mehaffey et al., 2010). Similar trends have been noted regarding the development of congenital heart defects (CHD). Specifically, children of heavy smoking mothers are at the highest risk for CHDs compared to children of other
smoking and non-smoking mothers (Malik et al., 2008; Lee and Lupo, 2013). Lastly, prenatal cigarette smoke has also been associated with increased risk for offspring asthma during childhood, adolescence, and adulthood (Häberg et al., 2007; Pattenden et al., 2006; Alati et al., 2006; Skorge et al., 2005). Taken together, these findings highlight the lasting consequences prenatal cigarette smoke has been shown to have on children’s cardiovascular health.

Experimentally, CS has been shown to drastically alter the profile and viability of the developing oocyte. Sobinoff et al. demonstrated that mice exposed to nose-only cigarette smoke for 12-18 weeks had ovaries that weighed significantly less than controls. These smoke exposed ovaries exhibited decreased numbers of primordial and primary follicles and increased apoptosis in secondary and antral follicles. In addition, the superovulated, CS-exposed mice produced roughly half the number of oocytes obtained from the controls. Correspondingly, authors conducted a small fertility study in which CS exposed mice took almost three times as many days to become pregnant and had smaller litter sizes overall (Sobinoff, et al. 2008). These data highlight the ability of cigarette smoke to be absorbed by the body where its thousands of components have the potential to reach the developing embryo and exert teratogenic effects. The individual constituents alone, such as the metals and metalloids discussed earlier, have been shown to be independently teratogenic.

Nicotine, the primary constituent and purpose for cigarette consumption, has been shown to be independently teratogenic. In a Wistar rat model, prenatal and perinatal exposure to nicotine were shown to induce mitochondrially-mediated pancreatic beta cell death in offspring which ultimately resulted in insulin resistance.
Prenatal nicotine exposure in mouse models has also been shown to induce hyperactivity in the resultant offspring (Pauly et al., 2004; Paz et al., 2007). These behavioral effects have been mirrored in recent C. elegans research. Rose et al. found that nematodes exposed to 30μM nicotine during larval development alone exhibited no significant differences in spontaneous movement when compared to control. However, nematodes that were exposed to 30μM nicotine during zygote and larval development, or zygote development alone, exhibited higher levels of spontaneous activity (Rose et al., 2013). The maintenance of this resultant phenotype across species suggests mechanistic conservation warranting further research.

Taken together, these findings highlight the teratogenic potential of CS as a whole and its individual constituents on multiple developing organ systems in the developing offspring.

1.6 The Fetal Origins of Health and Disease Hypothesis- Defining an Obesogen

British epidemiologist, David Barker, formulated and proposed what is now coined ‘The Fetal Origins of Health and Disease’ in 1990, after introducing evidence of a link between small size at birth and the development of cardiovascular diseases and comorbidities that persist into adulthood (Barker, Osmond, and Law, 1989; Barker, 1990; Barker, 1997). He proposed that the uterine environment was not impervious to external influence, and factors like maternal malnutrition and teratogens can reach and affect the developing embryo. As a result offspring are metabolically ‘programmed’ in such a way that they effectively survive the in utero stressor. However, the overall consequence of this new programming may increase susceptibility to metabolic disease
in later life. The paradigm has since permeated other research areas beyond that of cardiovascular health. The field of obesity in particular, has adopted the Fetal Origins of Health and Disease in the search for causal programmers of excessive and aberrant weight gain. Since an *in utero* exposure responsible for the development of any form of offspring disease is referred to as a teratogen, those that induce obesity were termed ‘obesogens’ (Grun and Blumberg, 2006).

Due to the markedly increased prevalence of obesity in the United States over the past several decades (Ogden, 2014), the National Institute of Environmental Health Sciences initiated a workshop employing scientists across multiple disciplines in a combined effort to review the literature and propose likely culprits for future pursuit in research (Thayer, Heindel, & Bucher, 2012). Just like teratogens, obesogens also span a wide variety of compounds from many different chemical classes, which similarly include the categories of estrogen mimics, biocides, metals and metalloids, and cigarette smoke. Obesogens are thought to function are by altering energy balance in the offspring such that they have a caloric surplus that is ultimately stored as lipids. Investigation of certain affected signaling pathways has provided some insight into their mechanistic role in the development of this phenotype.

1.7 Obesogenic Estrogen Mimics

1.7.1 Bisphenol-A

A great deal of information has been developed on the obesogenic potential of bisphenol-A. Initial work evaluating developmental exposure was aimed at addressing its potential toxicity to the developing reproductive system at low doses (vom Saal et al., 1998; Cagen et al., 1999). Studies of this nature were typically aimed at addressing the
noncanonical 'low dose effect' responsible in part for nonmonotonic dose response curves where the greater effects were observed at the lower end of the dose-response range (Vandenburg et al., 2012). Indeed, one of the earliest studies to highlight the effect on offspring weight gain was initially designed to address an earlier pubertal onset in CF-1 mice. Here, researchers confined an environmentally relevant dose of 2.4μg/kg/day BPA exposure to mice solely within the window of embryonic development between gestational days 11 and 17. Additionally all offspring were reared by foster mothers who did not have any treatment, further refining the exclusivity of the exposure model to the offspring. While researchers did find that prenatally exposed female offspring reached puberty sooner, body weights at postnatal day 22 were significantly increased in all exposed offspring across the board (Howdeshell et al., 1999).

Since then, BPA has demonstrated its obesogenic and lipid homeostatic alteration potential consistently across a host of model organisms ranging from the rodent to the invertebrate Drosophila. In 2001, Rubin et al. demonstrated obesogenic potential in male and female Sprague-Dawley rat offspring by exposing dams to 0, 0.1 (low), or 1.2 (high) mg/kg/day of BPA via drinking water from gestational day (GD) 6 through lactation (Rubin, 2001). Interestingly, for the female offspring a lower dose was found to have a more pronounced effect in significantly increasing body weight, and this was maintained well beyond 100 days of age (Rubin et al., 2001). A later study in Japan employing environmentally relevant doses (0, 1, and 10μg/mL BPA in drinking water) found similar effects. Specifically, at 30 days of age female ICR mice in the low and high dose groups showed significantly greater body weights than control offspring, while only males in the highest dose group displayed significantly increased body
weights. Interestingly, female offspring from the low dose exposure group showed a 123% increase in the amount of circulating leptin, a hormone that prompts satiety (Miyawaki et al., 2007). As no other significant changes were noted in even those other groups exhibiting significantly increased weight, the results suggest that BPA may exert its effects developmentally in a dose- and sex-dependent fashion. Furthermore, an analysis of food intake among offspring would have helped confirm what may have been leptin resistance in the females from the low-dose exposure group. In the fly, Drosophila melanogaster, BPA has also been shown to alter lipid storage and food intake in an exposure model encompassing egg through larval development. Flies exposed to the middle dose of 225ng/mg BPA through food exhibited significantly higher overall food intake. Additionally, flies exposed to 67.5ng/mg and 675ng/mg stored significantly more lipids after a 24-hour starvation period, inferring inhibited lipolytic capacity (Williams et al., 2014). Because BPA appears to have similar effects across different species, its mechanism of action too, may also be conserved. However, the exact dose-effects of BPA still need to be better refined. A recent study using similar maternal exposure window evaluated offspring effects in response to a higher dose of BPA has shown opposing effects. Here, administration of 50ng-50μg/kg/day via maternal diet to the A^vy mouse resulted in offspring hyperactivity and significant weight reductions when compared to control offspring (Anderson et al., 2013). 50μg/kg/day BPA is a higher dose than typically tested, and may be the reason for these different findings. In conjunction with the prior literature, the findings suggest that the effects of BPA on offspring weight are non-monotonic, where lower doses are obesogenic while higher doses are not.
Regarding causal signaling pathways, in 2009 Somm et al. (using an almost identical exposure model to Rubin et al., 2001) revealed alterations in the expression of genes involved in pro-adipogenic pathways from BPA exposed Sprague-Dawley rats. In this instance, only female offspring had increased body weights that persisted through 21 days of age. These offspring had significantly increased brown adipose and perigonadal white adipose tissue weights. Furthermore, in these tissues the mRNA expression of many pro-adipogenic genes such as PPAR\(\gamma\), SREBP-1C, C/EBP-a, and FAS were significantly upregulated, suggesting the pathways for lipid generation were more active in the developmentally exposed offspring than their controls (Somm et al., 2009).

Peroxisome-proliferator activated receptor gamma (PPAR\(\gamma\)) is a well-documented adopted-orphan nuclear hormone receptor implicated in adipocyte differentiation and proliferation that has been strongly implicated as one of the major molecular players in the development of obesity. Its lipogenic functions have even been demonstrated to function in other species. For example halogenated BPA analogs, commonly used as flame-retardants, have been shown to increase lipid storage and activate human PPAR\(\gamma\) in larval transgenic *Danio rerio*. Additionally, zebrafish exposed from 3 to 11 days post fertilization to 10nm and 100nm of tetrabromobisphenol-A (TBBPA) showed significantly increased levels of staining with the triglyceride-specific dye Oil Red-O, further highlighting the importance of this molecular pathway in BPA-mediated signaling cascades affecting lipid storage (Riu et al., 2014).
1.7.2 Diethylstilbestrol

Diethylstilbestrol is a second estrogen mimic that has both epidemiologic and experimental evidence supporting its obesogenic potential. One study as part of the Collaborative Perinatal Project examining women and children outcomes between 1959-1974 revealed that offspring of mothers who began using DES between the 3\textsuperscript{rd} and 4\textsuperscript{th} month of pregnancy were at almost 3 times the risk for overweight and obesity (Jensen and Longnecker, 2014). A more recent study highlights the potential dose-dependency and window of susceptibility for DES action on the developing fetus. Combining data from 4 separate cohorts of women and children, Hatch et al. revealed that prenatal exposure to DES conferred a slightly increased relative risk for obesity and overweight overall. However, the risk was gradually heightened with respect to the gestational age at which DES treatment began, being highest for those offspring who were exposed at 15 weeks of gestation. Furthermore, the risk for overweight and obesity was noticeably larger in daughters of mothers who had taken a lower dose (0.001mg/kg/day) of DES (Hatch et al., 2015). These findings mirror the low-dose obesogenic effects noted in the other estrogen mimic, BPA.

The experimental evidence of DES’s obesogenicity is divided. Mentioned earlier, administration of DES on GD13 in the Balb/C mouse was sufficient to induce the typical effect of cervical cancer in offspring, but these same offspring weighed significantly less at 5 months of age than their unexposed counterparts (Zulfahmi et al., 2013). However, it is important to note that for most women DES exposure likely occurred on a daily basis, as it was typically a treatment for morning sickness. Because of this, a chronic exposure model would better recapitulate the full teratogenic and obesogenic potential.
of the drug. Indeed, DES administration at 0.01 mg/kg/day in pregnant C57BL/6 mice from gestational day 12 through lactation was shown to significantly and sex-specifically induced weight gain in female offspring by 8 weeks of age. Developmentally exposed female offspring also showed significantly higher levels of circulating glucose and triglycerides (Hao et al., 2012). In vitro analyses revealed similar, supporting evidence of DES’ obesogenic potential. DES administered at 1μM and 10μM was shown to dose-dependently increased preadipocyte 3T3-L1 cell growth and lipogenesis as detectable via Oil Red-O staining. Furthermore, these cells also displayed increased mRNA transcript levels for molecular players involved in obesity including PPARγ and C/EBPa, reminiscent of changes seen in Somm’s work with BPA in the Sprague-Dawley rat in 2009 (Hao et al., 2012). These findings are in line with earlier evaluations by the Newbold lab, where low doses of maternally administered DES in mice were shown to induce increased offspring weight gain in both prenatal and neonatal exposure windows (Newbold et al., 2005).

1.8 Obesogenic Biocides

1.8.1 Tributyltin

Epidemiological evidence investigating the obesogenic potential of TBT is quite limited despite experimental evidence of its teratogenicity spanning the decade. One prospective study in Finland analyzing organotin content from 110 placentas and corresponding male offspring weight from birth to 18 months of age found that only TBT showed a significant effect. Specifically, offspring body weights corresponding to the highest group of placental TBT concentrations (>0.4ng/g) were significantly greater between 0-3 months of age. Associations were not maintained throughout 18 months of
age and no other effects were significant, however authors highlight the small sample size may have underpowered the study (Rantakokko et al., 2014).

Experimentally, TBT has been shown in vitro and in vivo in several model organisms to affect lipid homeostasis. In 2006, Grun et al. showed that TBT was capable of activating two pro-adipogenic nuclear hormone receptors, human RXRα and PPARγ. In 3T3-L1 cells treatment with TBT also induced lipogenesis as detectable via Oil Red-O staining. Interestingly, when they performed a model of in utero TBT exposure spanning gestational days 12-18 they did not find significant differences in weight gain at birth between exposed and control offspring. However, the prenatally TBT exposed male offspring did have significantly larger epidydimal fat pads compared to controls, suggesting a programmed predisposition towards storage of fat (Grun et al., 2006). Kirchner et al. postulated in 2010 that TBT worked prenatally to program mesenchymal stem cells to favor the generation of adipocytes, predisposing offspring to excessive weight gain before birth. To test this, pregnant C57BL/6 mice were first given a single oral dose of 0.1mg/kg TBT at embryonic day 16.5. When the offspring had reached 8 weeks of age, stromal cells were taken from their white adipose tissue and observed for their adipogenic potential. Cells from prenatally exposed mice generated at baseline 20% more lipids as indicated by Oil Red-O staining area when compared to cells from control offspring, suggesting that TBT programs developing stromal cells to prefer adipocyte differentiation and lipogenesis. Furthermore, when these prenatally exposed and control stromal cells were treated with TBT in vitro, these exposed cells nearly doubled their lipid accumulation compared to controls (Kirscher et al., 2010). While these studies highlight the obesogenic potential of TBT and elaborate those
mechanisms through which it may act, neither study examines offspring weight in adulthood specifically, indicating the need for further research in this area.

Lastly, while not an in utero model, TBT’s lipogenic potential has also been demonstrated in the transgenic zebrafish Danio rerio using a larval exposure window. Exposure to 0.1nm and 1.0nm during early development from 3 days post fertilization (dpf) significantly and dose-dependently increased lipid storage as detectable via Oil Red-O staining in larva at 11 dpf (Riu et al., 2014). These results highlight the importance of the hPPARγ ligand and the evolutionarily conserved signaling pathways through which TBT may act in the developmental programming of obesity.

1.8.2 Organophosphates

Several papers have been published regarding pro-adipogenic effects of organophosphates after neonatal exposure (Slotkin, Levin, and Seidler 2006; Adigun et al., 2010; Lassiter and Brimijoin, 2008), however few studies address the prenatal, obesogenic potential of organophosphates. Additionally, those studies that have addressed this window of exposure use different types of organophosphates that have resulted in differing, nonmonotonic dose-response curves for offspring weight.

Again, one of the earlier studies to provide such insight on this topic was originally intended to address questions of altered reproductive development. Pregnant rats were administered Fenitrothion at doses ranging from 0-25mg/kg/day from GD 12 to GD21. In both male and female offspring at PND21, a non-monotonic U-shaped dose-response in body weight is noticeable. However while the offspring in both sexes at the highest exposure level (25mg/kg/day) do weigh about 4.5g more than their control counterparts, the difference does not appear to be significant (Turner et al., 2002).
2008, Lassiter and Brimijoin demonstrated in the Long-Evans rat that maternal exposure to 2.5mg/kg of the organophosphate pesticide chlorpyrifos with an earlier exposure window of GD7 through weaning, induced sex- and dose-specific changes in weight gain of the offspring. The exposure did not alter birth weight in offspring, but after the weaning period males specifically were shown to gain significantly more weight than their unexposed male counterparts. Further analysis using an identical exposure window with 4 doses ranging from 0 to 4mg/kg directed at solely the male offspring revealed an inverted-U shaped dose-response curve where males exposed developmentally to 2.5mg/kg exhibited the highest overall body weight (Lassiter and Brimijoin, 2008). Taken together, these results highlight potential windows of susceptibility and differences in obesogenic potency between organophosphate pesticides.

1.8.3 Triclosan

Evidence for the obesogenic potential for the common biocide triclosan is very limited. One epidemiological study investigated the direct effects of triclosan on BMI and found that persons falling into the middle ranges of triclosan exposure (10.6-58.3ng/mL urine) showed the most robust increases in BMI compared to those with higher exposure levels (Langkester et al., 2013). The study however does not account for developmental exposure, which is central to the obesogen hypothesis. Currently only one study presenting such information is the experimental evaluation of prenatal triclosan on pubertal developments in rats by Rodríguez and Sanchez in 2010. Interestingly, rats that were exposed prenatally and throughout lactation to 1.0-50mg/kg/d triclosan via drinking water showed reduced body weights compared to
vehicle control offspring at PND20. However, on the first day of VO (vaginal opening, observed within PND30-PND38), considered the start of pubertal onset in the rat, these same developmentally exposed rats showed significant increases in body weights compared to control treated offspring. Furthermore, these weight differences are further exacerbated in offspring that continued to receive triclosan treated drinking water until VO (Rodríguez and Sanchez, 2010). These particular findings have gone relatively unnoticed by others citing the study, but are especially salient in suggesting that early developmental exposures to triclosan may function in concert with the hormonal changes incurred when offspring enter adulthood to induce aberrant weight gain.

1.9 Obesogenic Metals and Metalloids

The epidemiologic evidence appears to contradict the notion of cadmium’s and arsenic’s obesogenic potential, as several recent studies report that prenatal exposure to these agents are associated with reduce birth weight and size, or reduced weight in childhood (Rollin et al., 2015; Delvaux et al., 2014; Kippler et al., 2013). However, it is important to note that these same anthropometric endpoints are not continuously evaluated through the pubertal or adulthood stages of life, which Rodriguez and Sanchez have previously demonstrated may be a trigger for exposure-dependent weight gain despite an initial reduction in birth weight (Sanchez and Rodríguez, 2010). It is also important to note that direct exposure to either of these agents has been linked to altered metabolism and glucose homeostasis. Infants and their diabetic mothers for example, have higher circulating levels of blood cadmium and arsenic compared to infants from nondiabetic mothers (Kolachi et al., 2011). While authors highlight that these mothers may be more in contact with environmental teratogens, similar reports of
cadmium and diabetic state have been reported in earlier studies. Researchers examining adults as part of the National Health and Nutrition Examination Survey III found that those who had higher urinary cadmium (>1 μg/g creatine) were at a significantly increased risk for impaired fasting glucose and diabetes (Schwartz, Il'yasova, and Ivanova, 2003). More recently, researchers in South Korea found sex-specific relationships between blood cadmium levels and development of metabolic syndrome in men. Metabolic syndrome indicates the patient is afflicted with 3 of the following 5 ailments: abdominal obesity, glucose intolerance, hypertension, high circulating triglycerides, and low high-density lipoprotein cholesterol (NIH, 2015). Specifically, men who had double the level of circulating cadmium were at a 23% increased risk for developing metabolic syndrome (Lee and Kim, 2012). The current information on the direct effects of metal and metalloid exposure on metabolism provides interesting opportunities for further research on obesogenic potential from a developmental standpoint and the mechanisms through which these agents may act.

1.10 Obesogenic Effects of Developmental Cigarette Smoke Exposure

Prenatal CS has a history of inducing low birth weight in the offspring, but increasing the risk of childhood and adulthood obesity in studies conducted worldwide. A meta-analysis in 2008 examining 14 studies found that children of smoking mothers showed a significantly increased risk for overweight even after adjustment for maternal confounders. These results indicate the effect on offspring weight is likely due to the exposure and not postnatal lifestyle factors (Oken, Levitan, and Gilman, 2008). Because low birth weight is a common effect of prenatal smoking, research was also conducted to investigate whether or not offspring obesity was a result of “catch up
growth”. Interestingly, offspring of smoking mothers showed higher overall BMIs irrespective of initial birth weight, suggesting aberrant developmental metabolic programming to be the more likely causal culprit in lieu of the ‘catch up growth’ hypothesis (Beyerlin et al., 2011).

Clinical studies have corroborated the obesogenic effect of CS via MRI and bone densitometry scanning, where analyses have shown that prenatally smoke exposed offspring possess larger depots of subcutaneous and intra-abdominal body fat (Leary et al., 2006; Syme et al., 2010). Interestingly, one study from Canada revealed that prenatally smoke exposed offspring with increased body fat composition also had smaller amygdalas, suggesting a concomitant developmental alteration of the neural circuitry mediating food intake (Haghighi et al., 2013).

Other studies have revealed dose-effects of prenatal CS. Specifically, children of mothers who were heavier smokers during pregnancy were at an increased risk for developing obesity than those children of mothers who were classified at ‘light’ smokers (Koshy, Delpisheh, and Brabin, 2010; Harris, Willett, and Michels, 2013). Additionally, cessation by the first trimester ameliorated this risk to some extent, suggesting windows of susceptibility through which this multifaceted obesogen may act (Harris, Willet, and Michels, 2013).

Experimentally, prenatal cigarette smoke has been shown to increase weight gain and alter lipid homeostasis in the mouse. Specifically, pregnant B6C3F1 hybrid dams were exposed to whole body CS or normal air from GD4 through parturition. The resultant offspring were raised on a standard diet for approximately 12 weeks, after which they continued on the standard diet or were switched to a high fat diet containing
42% calories from fat for 2 weeks. Interestingly, differential effects on body weight were dependent on sex and diet. On the standard diet, prenatally smoke exposed female offspring weighed significantly more than their unexposed counterparts and exhibited higher circulating HDL and LDL cholesterol, while there were no significant differences noted among the males. When fed the high fat diet for two weeks however, prenatally smoke exposed male offspring weighed significantly more and had corresponding higher circulating HDL cholesterol than unexposed males (Ng, 2009). A second study using the Balb/c mouse revealed that developmental exposure to cigarette smoke significantly increased offspring total body weight and fat pad weight while simultaneously altering glucose homeostasis. Furthermore, prenatally smoke exposed offspring were shown to weigh more at 12 weeks while consuming fewer calories overall (Chen et al., 2011). These data provide experimental support of CS’s obesogenic effects, however the individual constituents have also been shown to alter lipid metabolism and glucose homeostasis as well.

Developmental exposure to nicotine for example, has been shown to have effects on offspring weight and glucose homeostasis. Exposure to 1mg/kg body weight of nicotine from a period of preconception through weaning in the Wistar rat has been shown to increase offspring body weight and total fat pad weight (Gao et al., 2005). A similar rat model exposing pregnant dams to nicotine throughout pregnancy and lactation induced increased body weights, insulin resistance, and alternations in glucose metabolism at 26 weeks of age in developmentally exposed offspring (Holloway et al., 2005). These results seemed to indicate the manifestation of a type 2 diabetic
phenotype, which was later shown by the same group to be the result of mitochondrially mediated pancreatic beta cell death (Bruin et al., 2007; Bruin et al., 2008).

Benzo-\(\alpha\)-pyrene (BAP) is a second major constituent of cigarette smoke that has been shown to alter lipid homeostasis, however to date, not from a developmental standpoint. Injection of C57BL/6 mice with 0.5mg/kg every 2 days for approximately 2 weeks resulted in significantly increased body weights of treated mice compared to controls. Furthermore, exposed mice exhibited higher levels of circulating cholesterol and triglycerides (Layeghkhavidaki et al., 2014). While not from a developmental standpoint, these findings in conjunction with BAP’s ability to reach the developing oocyte and embryos (Zenzes et al., 1995; Zenzes et al., 1998) highlight the potential effects it could have on early lipid metabolism and metabolic programming.

1.11 The Role of Peroxisome Proliferator-Activated Receptors in Obesity

1.11.1 An Introduction to PPARs and Their Implication in Obesity

There are three types of PPARs- alpha, beta (also known as delta) and gamma, that function in the human body and in part, modulate lipid homeostasis. These PPARs are expressed in different tissues and have even seemingly opposing functions. Work by Escher et al. revealed in 2001 the differences in tissue distribution between the three PPAR isoforms in the Sprague-Dawley rat (Escher et al., 2001).

PPAR\(\gamma\) specifically may exist as two isoforms, PPAR\(\gamma\)1 and PPAR\(\gamma\)2 (Zhu et al., 1995). Escher et al. revealed that PPAR\(\gamma\)1 is expressed at low levels in several tissues, but at very high levels in both BAT and white adipose tissue (WAT). PPAR\(\gamma\)2 however was expressed mainly in the BAT and WAT. In the liver, PPAR\(\gamma\) functions ultimately stimulate lipid storage and gluconeogenesis. In the adipose tissue, PPAR\(\gamma\) functions to
stimulate adipogenesis and remodeling. The resulting uptake of fatty acids and glucose for these anabolic processes is thought to aid in insulin sensitization in skeletal muscle (Ahmadian et al., 2013). In these tissues, PPARγ may work with other factors that promote and perpetuate its expression and action in these tissues. The uptake of fatty acids and promotion of insulin sensitivity are two major means by which PPARγ agonists such as the thiazolidinedione class of drugs function therapeutically to ameliorate insulin resistance in patients with type 2 diabetes. However, stimulation of this nuclear hormone receptor in particular also results in the undesirable effect of weight gain (Larsen, Tourbro, ad Astrup; 2003).

PPARα has been shown to be expressed in the liver, kidney, heart, lung, and brown adipose tissue (BAT) (Escher et al., 2001). In contrast to PPARγ, PPARα typically functions in these organs to stimulate catabolic processes such as lipid breakdown via fatty acid oxidation. Impaired PPARα signaling has been shown to result in fatty liver in mice (Wang et al.; 2010). PPARδ performs analogous functions, and was shown to be the most widely expressed among the 22 organs analyzed (Escher et al., 2001). Overexpression of PPARδ signaling has been shown to provide protection against obesity, even in the presence of a high fat diet. Furthermore fibrate drugs such as benzafibrate, serving as agonists of both PPARδ and PPARα, encourage the generation of mitochondria (Wang et al.; 2010). Collectively, the literature highlight that while PPARγ stimulation leads to fatty acid uptake and weight gain, PPARδ and PPARα stimulation may be protective in promoting fatty acid oxidation.
1.11.2 Environmentally Implicated PPARγ

Mentioned earlier, three of the previously highlighted obesogens have been shown to increase PPARγ expression and signaling in either in vitro and/or in vivo studies. These studies bring to light the very real effects environmental teratogens can exert on those same molecular signaling pathways that are implicated in the disease of human obesity.

Hao et al. demonstrated in vitro with the 3T3-L1 preadipocyte cell line that DES administration increased PPARγ expression and expression of its target genes C/EBP and LPL in a dose-dependent fashion. Furthermore, these cells also exhibited significantly higher levels of stored triglycerides when stained with Oil Red-O. In vivo they demonstrated that direct administration of 0.5mg/kg to 6-week old male C57Bl6 mice was similarly sufficient to significantly increase expression of PPARγ and its target LPL in the adipose tissue (Hao et al., 2012). While they also demonstrate that perinatal administration of 0.01mg/kg/day of DES was sufficient to increase female offspring weight at 8 weeks, expression levels of PPARγ and its target are not included. Since PPARγ has proven to be expressed in embryogenesis (Jain, 1998; Barak, 1999), it may be beneficial to further investigate the expression of this receptor in the adipose tissue of offspring exposed to DES in utero, perhaps as a time-course study throughout the process of weight-gain.

Grun et al. in 2005 demonstrated that the organotin TBT was a potent stimulator of adipogenesis via PPARγ-mediated pathways both in vitro and in vivo. Using a Cos7 cell line transiently transfected with GAL fusions to several different nuclear hormone receptor LBDs they were able to show that administration of TBT specifically induced
activity of PPARγ and RXRα. Further evaluations in vitro using the 3T3-L1 preadipocyte cell line revealed that TBT treatment also greatly increased lipid production and storage as detectable via Oil Red-O staining. Lastly, to assess the obesogenic effects of TBT in vivo, they administered 0.5mg/kg/day to pregnant C57BL/6 mice from GD 12 through parturition. In newborn mouse offspring exposed in utero to TBT, Oil Red-O staining was significantly elevated in mammary and inguinal adipose sections, inferring either increased lipid storage or generation of adipocytes. Additionally, some newborn pups were also cross-fostered to untreated dams for 10 weeks. While body weights were not significantly different between prenatally treated and control offspring, TBT-exposed male offspring had larger epidydimal fat pads. As mentioned previously, these data indicate that developmental exposure to TBT may be programming offspring to preferentially generate and store lipid tissue despite significant changes in weight gain (Grun et al., 2006). Further analysis following these offspring into later adulthood or under the influence of a higher fat, ‘Western’ diet may be useful at revealing increased susceptibility for weight gain and the role of PPARγ and TBT in the context of the postnatal nutritional environment.

Riu et al demonstrated in 2014 interesting effects of TBT and the halogenated BPA analogs TBBPA and TCBPA on human and zebrafish PPARγ activity. Using a zebrafish transgenic for human PPARγ, Tg(hPPARγ-eGFP), zebrafish embryos were exposed for 24 hours to 10nM or 100nM rosiglitazone, 100nM or 1μM TBBPA, 1μM TCBPA, or 0.1% DMSO vehicle. After the 24 hours, strongest GFP expression was noted in rosiglitazone treated embryos, and to a lesser extent those treated with either TBBPA or TCBPA. Because zebrafish possess their own homolog of PPARγ, they also
wanted to investigate the extent to which these agents promoted activity of this receptor across species. Similar to prior work, they first generated a bioluminescent reporter cell line for zPPARγ activity, then treated these cells with a range of concentrations of TBT, TCBPA, TBBPA, and various hPPARγ-agonizing thiazolidinediones. Interestingly, zPPARγ did not respond to rosiglitazone or TBT. They did note however a weak stimulatory effect by TCBPA and TBBPA. Lastly, zebrafish exposed during larval development to TBBPA, TCBPA, and TBT also showed stronger staining with Oil Red-O compared to their vehicle-exposed counterparts (Riu et al., 2014). The fact that zebrafish exposed to these putative obesogens stored more lipids despite weak zPPARγ in vitro indicates that these agents, while capable of modulating the lipogenic PPARγ pathway, may be acting via additional complementary pathways to achieve this phenomenon of weight gain.

In conclusion, PPARγ is expressed during embryogenesis (Jain et al., 1998), posing the possibility that it may function during early development to induce pro-adipogenic pathways and continue to function in the manifestation of offspring obesity in later life. Due to the fact that PPARγ is a demonstrated major player in the development of obesity as a master regulator of adipogenesis, the search for functional homologs in other model organisms should maximize our understanding of it’s mechanistic role under the influence of environmental obesogens.

1.11.3 PPARγ Structure

Alongside the other PPARs, PPARγ is a member of the nuclear hormone receptor (NHR) family. As an NHR, PPARγ contains several regions typical to this receptor family: First, a highly conserved C-terminal domain in which the Ligand Binding
Domain (LBD) and ligand-dependent AF-2 transactivation region is located. The ligand-binding domain yield 13 alpha-helices and one beta-sheet to form a ligand binding “pocket” (Berger and Moller, 2002). Second, a DNA-binding domain, which is also highly-conserved encoding two zinc-fingers that bind to PPAR-response elements (PPREs). And third, a variable N-terminal A/B domain in which the ligand independent AF-1 transactivation region is located (Berger and Moller, 2002; Robinson-Rechavi, Garcia & Laudet, 2003).

1.11.4 PPARγ Ligands

It has been shown that certain unsaturated fatty acids, such as linoleic and arachidonic acid, may act as ligands for PPARγ (Berger and Moller, 2001). Although the identification of a specific endogenous ligand for this receptor is still absent, rendering it an ‘adopted orphan’ receptor (Xu and Li, 2008).

The thiazolidinedione category of drugs, classically used to treat Type-2 diabetes mellitus, comprise well-known, potent pharmacologic ligands with great specificity for PPARγ (Lehmann et al., 1995). Their therapeutic benefit is primarily due to their ability to increase insulin sensitivity and moderate glucose homeostasis. However, drugs in this category (e.g. troglitazone and rosiglitazone) have been controversial for treatment purposes due to side effects including liver toxicity, cardiovascular events, and bone loss (Derosa & Maffioli, 2012; Ahmadian et al., 2013).

1.11.5 Specific PPARγ Lipogenic Functions

PPARγ forms an obligate heterodimer with the Retinoid X Receptor (RXR) which is typically inactive, bound to co-repressors in the absence of a ligand. Ligand binding to the PPARγ-RXR induces conformational changes in the proteins and promotes
recruitment of transcriptional co-activators that allow it to bind its PPRE in the DNA within the nucleus (Montanari et al., 2008). As mentioned earlier, many pathways involved in adipogenesis are transcriptional targets for upregulation by PPARγ. In the WAT for example, PPARγ act to increase expression of acyl-CoA synthetase, phosphoenol pyruvate carboxykinase (PEPCK), lipoprotein lipase (LPL), and CAAT-enhancer binding protein alpha (C/EBPα) (Tontonoz, 1995; Berger and Moller, 2001; Ahmadian, 2013). While acyl-CoA synthetase is known to function in catabolic pathways, it may also be employed in the anabolic process of lipogenesis. PEPCK catalyzes the rate-limiting step in gluconeogenesis and adipose glyceroneogenesis (Tontonoz et al., 1995). LPL is involved in the hydrolysis of triglyceride that aids in cellular fatty acid uptake by cells (Goldberg, Eckel, and Abumrad, 2009). C/EBPα is another type of transcription factor that functions to increase the expression of lipogenic genes, promoting adipocyte differentiation (Freytag, Peielli, and Gilbert, 1994; Rosen and Spiegelman, 2001). PPARγ activity can also modulate physiological changes in behavior that have implications in the development of obesity. PPARγ activation reduces the expression of leptin expression in WAT. Leptin is a hormone secreted by adipocytes that reduces food intake. Activation of PPARγ by administration of a thiazolidinedione agonist in the Sprague-Dawley rat for example, was shown to decrease the expression of leptin, increase adiposity, and increase food intake (De Vos et al., 1996).

### 1.11.6 PPARγ Conclusions

PPARγ is an interesting avenue for future research regarding the signaling pathways utilized by obesogens. Finding a functional homolog for this receptor in a
more straightforward *in vivo* model such as *C. elegans* may help maximize our understanding of its mechanistic role in obesity, its natural ligands, and aid in the discovery of potential therapeutics.

### 1.12 Caenorhabditis elegans Origin in Research and Basic Biology

*Caenorhabditis elegans* is a small soil nematode, initially adapted by Sydney Brenner in the mid 1960s as a model organism to study neurodevelopment (Brenner, 1974; Wood, 1988). The more commonly used strain is the N2 Bristol strain, named because Brenner had established in 1964 a ‘N2’ line of *C. elegans* from a stock that had been initially found outside of Bristol, England (Riddle, 1997). One of the primary advantages of this model organism is the ease of maintenance and manipulation. *C. elegans* may be cultured at room temperature, ranging from 16-25°C and feed upon a diet of *Escheria coli*, a bacteria readily accessible to most biological laboratories (Brenner, 1974).

A second advantage is the well-characterized nature of this model organism. Indeed, over two decades were spent investigating every aspect of this animal from its genome, to its cell division throughout its lifecycle (Wood, 1988). *C. elegans* have a mapped genome, spanning approximately 100 million base pairs (C. elegans Sequencing Consortium, 1998). Spanning 6 haploid chromosomes, *C. elegans* are present as either hermaphrodites homozygous for XX in their sex chromosomes, or males arising through spontaneous nondisjunction resulting in an XO genotype. Hermaphrodites produce both oocytes and sperm and are thus capable of self-fertilizing. Males produce only sperm, and may fertilize hermaphrodites during the later stage of larval development (Wood, 1988).
Fertilized embryos develop in the uterus of the hermaphrodite and are typically laid as eggs in the gastrula stage (Sulston et al., 1983). *C. elegans* pass through 4 larval stages after hatching - L1, L2, L3, L4, before entering adulthood. The transition from egg to gravid egg-laying adult takes approximately two days at 25°C (Byerly, Cassada, and Russel, 1976). The lifecycle of *C. elegans* is temperature-dependent, at a higher temperature like 25°C the average lifespan is about 9 days, while at lower temperatures of 16°C the average lifespan can be as many as 23 days (Klass, 1977). In the absence of sufficient food, or the condition of overcrowding, it is possible for *C. elegans* L1-stage larvae to bypass the L2- and L3-stages to enter a period of stasis known as the dauer stage. Expending minimal energy, they may survive in the dauer stage without food for several months. When conditions for growth are once again optimal, *C. elegans* may exit the dauer stage and reassume development as an L4-stage larva (Riddle and Albert, 1997). L4-stage larvae are easily discernable from other larval staged nematodes as they possess an abdominal ‘clear patch’ that is the developing vulva for the adult nematode (Sulston et al., 1988). About a millimeter in size once adult, hermaphrodites possess a finite 1031 somatic cells and males possess a finite 959 somatic cells (Wood, 1988; Sulston et al., 1988). Furthermore, the lineage of these cells has been mapped from conception to adulthood (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983). The *C. elegans* body is transparent further facilitating the observation of changes that may arise from exertion of external influence. Detailed below are specific pertinent advantages that the biology of the nematode lends to the research areas of Reproductive Toxicology and Obesity.
1.13 *Caenorhabditis elegans* Utility in Reproductive Toxicology Research

Hirsh et al. detailed the development of the reproductive system of *C. elegans* in the mid-1970’s. *C. elegans* gonadal development begins from four primordial germ cells in the L1-stage larva. Over a span of approximately two days at 25°C, these cells proliferate generating two gonadal arms that protrude in opposite directions from the center of the nematode. Extending approximately 340nm in the adult *C. elegans*, these gonadal arms loop around to generate a region of oogenesis that pushes oocytes towards the spermatheca to be fertilized. After fertilization, these embryos undergo early cellular divisions before they are laid as eggs surrounded by a protective chitinous shell. At 25°C an adult hermaphrodite will begin to self-fertilize around 37 hours of age and begin egg-laying at hours 45-46. At this temperature, the average self-fertilizing hermaphrodite can lay up to approximately 180 eggs, turning over the volume of their gonad in a little under 6.5 hours (Hirsh, Oppenheim, and Klass, 1976).

The short lifecycle, high progeny count, and physical transparency of this organism are particularly well suited to the execution of reproductive toxicity studies. For instance, it is possible to gather information on embryonic percent viability in response to an exogenously applied agent by counting the number of eggs laid and the number of hatched larvae in a given period of time. This data collection also provides useful information on the overall resultant parental fertility (ability to generate an embryo) and fecundity (the absolute number of live offspring produced). Since the hallmarks of *C. elegans* growth were temporally established by Hirsh in 1976, it is also easy to detect chemicals that cause a developmental delay by monitoring onset of
reproductive organ development (such as the clear patch to vulva transition) or onset of egg-laying.

Additionally, individual *C. elegans* gonads may be dissected out and DAPI stained to reveal morphologic changes in the progression of cells through the germline of pollutant-exposed nematodes (Allard and Colaiácovo, 2010). More recently, the researchers have taken advantage of the nematode’s transparency in the generation of a high-throughput screen for potentially aneuploid promoting compounds. In 2013 Allard and Colaiácovo used a *Pxol::gfp* transgenic strain of *C. elegans* for this very purpose. Commonly referred to as “Green eggs and him”, the transgenic worm contains a green fluorescent protein (GFP) inserted downstream of the male-specific promoter for *xol-1*, a gene normally turned in males who are haploid for the sex-chromosomes (XO). When males arise through spontaneously meiotic nondisjunction of the sex chromosomes, the *xol-1* gene and therefore its promoter are active, in turn activating expression of the transgenic GFP (Nicoll, Akerib, and Meyer 1997; Kelley et al., 2000). Aneugenic compounds are known to increase meiotic nondisjunction across all chromosomes, however in *C. elegans* application of these agents would also be expected to increase the meiotic nondisjunction in the sex chromosomes as well, ultimately generating more male offspring. Realizing the utility of the *Pxol::gfp* strain in conjunction with the natural biology of the nematode, Allard and Colaiácovo exposed L4-staged *C. elegans* of this strain to 47 chemicals from the ToxRef database for either 24 or 65 hours to evaluate aneugenic potential. Compounds that were regarded as more aneugenic would result in higher frequencies of male GFP-expressing embryos, detectable via the COPAS BioSorter large particle flow cytometer specialized for *C.
Interestingly, and perhaps most importantly, this screen confirmed the aneuploidy promoting chemicals previously classified as reprotoxic to mammals, but also detected new found aneuploid induction from other compounds that had no prior record of mammalian reprotoxicity (Allard and Colaiácovo, 2013). These findings highlight how the straightforward biology of *C. elegans* has been harnessed for the execution of studies ranging from the most fundamental evaluations of reproductive toxicity, to those designed to be high-throughput screens capable of efficiently evaluating hundreds of chemicals.

1.14 *Caenorhabditis elegans* Utility in Obesity Research

*C. elegans* are useful to the field of obesity research for a number of reasons. There is a notable degree of homologous genes involved in lipid metabolism and related pathways conserved between humans and *C. elegans*. Additionally, energy balance is similarly measurable in *C. elegans* via a variety of techniques designed to infer energy intake and energy expenditure. In addition, other mutant alleles, such as tubby homozygous null allele and tub mutants, found in mammals that result in obesity, also cause an obesity phenotype in *C. elegans* with similar mutations in their respective homologs, such *tub-1*.

1.14.1 Lipid Storage and Its Measurement in *C. elegans*

*C. elegans* lack adipocytes, however they do store excess energy in the form of lipid droplets within their intestinal and hypodermal cells. Despite this, one major advantage of using *C. elegans* is the amount of relevant gene homology between the nematode and humans. Zhang et al. in 2013 compiled a database of 471 *C. elegans* genes involved in lipid metabolism that spanned 16 relevant pathways including fatty
acid biosynthesis and elongation. Using the bioinformatics process of phylogeny tree construction and analysis, it was revealed that 370 (78.56%) of these lipid metabolic genes were conserved in humans. Furthermore, 94 of these *C. elegans* genes corresponded to human orthologs associated with metabolic disease (Zhang et al., 2013). *C. elegans* also possess homologs of major players involved in the development of human obesity, for example SREBP and C/EBP. Similarly, they also employ some nuclear-hormone mediated pathways involved in the mobilization and metabolism of lipid stores similar to those seen in mammals. NHR-49 for example is thought to function analogously to the mammalian anti-obesogenic PPARα, an NHR in the same subfamily as PPARγ. NHR-49 knockdown leads to increased fat storage in *C. elegans*, a phenomenon noted in PPARα knockout mice (Pathare et al., 2012). However, evidence for functional homologs in *C. elegans* for other major adipogenic NHRs like PPARγ, is still lacking and warrants further research.

A second major highlighted advantage of *C. elegans* as a model for obesity is the visualization of lipid storage at “single-cell resolution” (Mak, 2012). Oil Red-O is a stain that is typically chosen because of its high degree of specificity for lipid droplets in fixed nematodes, a fact established, tested, and replicated in the literature (Ramirez-Zacarias et al., 1992; O'Rourke et al., 2009; Yen et al., 2010). *C. elegans* has proven to show differential lipid storage with this stain in response to externally applied conditions including drugs, neurotransmitters, and other chemicals. For example, dopamine has been shown to reduce lipid storage in *C. elegans* in a seemingly dose-dependent fashion (de Almeida Barros et al., 2014). Dopamine treated nematodes were then subjected to a battery of lipid quantification tests, the results of which appear to confirm
the prior protocol comparison by Yen 2010. Specifically, nematodes were stained with vital (live) dyes including Nile Red and BODIPY, fixative dyes including Sudan Black and Oil Red-O, and CARS imaged (coherent anti-stokes Raman imaging). CARS is a microscopy technique that allows the quantification of fat stores based on the amount of molecular vibration of the attached CH$_2$ groups. It provides information on not only the amount of lipid stored, but can also be used to characterize the type of lipid (Yen et al., 2010). Because of these factors, it is regarded as a highly specific tool capable of yielding, among other techniques, more reliable lipid storage data. However, the technique is still limited in terms of accessibility and cost feasibility. In the 2014 study by de Almeida Barros et al., fixative staining with Oil Red-O dye correlates CARS’s data trend strongest when compared to the other types of fixative and vital stains.

*Caenorhabditis elegans* store their excess energy exclusively as triglyceride droplets in their intestine and hypodermis. Enzymatic colorimetric-coupled triacylglyceride (TAG) assays are also sometimes also adapted to *C. elegans* research in an attempt to more finely quantify this lipid storage. The assay couples the enzymatic liberation of the triglyceride’s glycerol backbone to the generation of a purple quinonimine dye detectable at 540nm via plate reader. The two sequential subsets of reactions proceed after addition of an enzymatic mixture to the sample lysate. In general, lipase acts to break the fatty acids from the glycerol backbone of the triglyceride. After this step, glycerol kinase phosphorylates this backbone to generate glycerol phosphate. The final step in this first arm of reactions uses glycerol phosphate oxidase to oxidize glycerol phosphate, generating dihydroxyacetone phosphate and hydrogen peroxide. In the second arm of the assay, the generation of this hydrogen peroxide is coupled via
peroxidase to generate a specific purple quinonimine dye (Cayman Chemical Company, manufacturer’s brochure).

Hench et al. in 2011 demonstrated that a similar TAG assay was able to correlate Oil Red-O staining data, where daf-2 mutant C. elegans stored significantly more lipid droplets than their control, N2 wild-type counterparts (Hench et al., 2011). The insulin receptor homolog, daf-2, is a known inhibitor of the lipogenic daf-16 which functions to promote stress resistance (Ashrafi; 2006). However, it should be noted that these kits are typically tailored for use with cell culture, serum, or organ tissue, not nematode samples. As C. elegans have a tough, thick cuticle that is especially difficult to break apart, special care should be taken to ensure proper homogenization of the sample prior to execution of this assay.

1.14.2 Energy Balance and Its Measurement in C. elegans

Energy balance may be best described as the difference between energy intake and energy expenditure. Where a net negative energy balance is typically associated with weight loss as it results in a calorie deficit; and a net positive energy balance is associated with weight gain as it results in a calorie surplus that is ultimately stored as triglycerides.

Pharyngeal pumping rate is a common assay used to infer energy intake in C. elegans. A pharyngeal pump may be best visualized in C. elegans via grinder movement (Avery & Shtonda, 2003). Day 1 adult N2 C. elegans have literature reported pharyngeal pumping rates when fed OP50 (or nutritionally equivalent strains) of approximately 250-300 pumps per minute (PPM) in multiple studies (Huang et al., 2004; Raizen, 2012; Soukas et al., 2009). This procedure may be performed on a
standard research stereomicroscope. The pharyngeal pumping assay is a common tool that is used when establishing a metabolic profile, and has also been used to characterize nematodes with respect to influential factors like mutant genotype, food quality, age, time of day, and exposure to drugs and chemicals. For example, *C. elegans* have been shown to increase pharyngeal pumping rates when fed the standard quality strain of *E. coli* OP50, in comparison to higher calorie bacterial strains like HB101 (Soukas et al., 2009). In *C. elegans*, the day age of adulthood has been shown to significantly alter pharyngeal pumping rate, peaking around day 3 and declining thereafter (C. Huang et al., 2004). Circadian rhythm has also been evaluated with respect to pharyngeal pumping rates. Specifically, a sigmoidal trend appears to be present lasting approximately 24 hours with lowest rates reported at the initiation of the 12-hour dark cycle (Migliori et al., 2011). These factors such as diet and time of day should be taken into account and controlled for when executing a study, since they have been shown in prior work to influence pharyngeal pumping rate independent of the externally applied conditions.

The body bending frequency assay, also called the ‘thrashing assay’, measures the activity level in a given nematode and may be one method used to infer energy expenditure. One ‘thrash’ is typically defined as a change in the direction of the body bending at the midline (Miller et al., 1996). Adult N2 *C. elegans* under standard conditions have greatly varied reports on the average body bending frequency, ranging from 120-230 thrashes/min (TPM) (Buckingham & Sattelle, 2009; Cohen et al., 2009). The assay has been used in conjunction with other behavioral tests, and has been shown to be superior in the characterization of *C. elegans* phenotype with respect to
neural signaling, metabolism, and drug effects. For example sulfonamides, generally used as an antibiotic, have also been shown to significantly lower body bending frequencies in exposed nematodes for not only the parental, but also the unexposed offspring. Body bending frequency was asserted as one of the most sensitive behavioral assays in this same study (Yu et al., 2013).

Oxygen consumption is a second technique that may be used to infer aerobic energy expenditure in *C. elegans*. Oxygen consumption is typically measured with a Clark-type microelectrode. This tool has been commonly used to quantify overall energy expenditure in well-established labs using *C. elegans* as a primary model system. The rate of oxygen consumption has been shown to vary with respect to experimental factors such as mutant genotype, diet, neurohormone regulation, and circadian rhythm. Soukas et al. found N2 *C. elegans* were more metabolically active on a high quality, nutrient rich food strain of *E. coli* HB101 than the standard OP50. In addition, *rict-1* mutants exhibited less oxygen consumption overall (Soukas et al., 2009). Regarding neurohormone regulation of metabolism, Cohen et al. found that *flp-18* mutant *C. elegans* store roughly 1.5-2X as much lipid compared to WT. Furthermore, a difference in physical activity was not to blame, as body bends/min and pharyngeal pumping rate were not noticeably different between the two groups. Ultimately, evidence of reduced oxygen consumption rate by ~33% inferred altered aerobic metabolism as a causal pathway (Cohen et al., 2009). Lastly, *C. elegans* have also shown to exhibit circadian patterns of activity when subjected to 12-hour light/dark cycles, with peak consumption occurring during the middle of a 12 hour dark period (Migliori et al., 2011).
1.15 Objective, Hypotheses, and Aims

The objective of this work was to accurately model the Fetal Origins of Health and Disease within the context of human obesity using the nematode, *Caenorhabditis elegans*. Obesogens are a type of teratogen, and teratogens are by nature repro- and embryo-toxic. It is therefore hypothesized that chronic exposure to these agents in *C. elegans* will have adverse effects on fertility and reproduction as revealed via changes in egg-laying, hatching, and embryonic percent viability. Secondly, it is hypothesized that *C. elegans* exposed to sub-embryonic lethal doses of putative obesogens during early embryonic development will have an increase in the amount of stored lipids once they have reached adulthood. The increase in stored lipids may manifest through alternations in energy balance (energy intake - energy expenditure) and aberrant molecular lipogenic signaling (e.g. nuclear hormone signaling). For an illustrated schematic please see Figure 1.1. Lastly, it is well known that the nuclear hormone receptor PPARγ is a major documented molecular player in the development of obesity. It is hypothesized that a combined bioinformatic and functional analysis may reveal a functional *C. elegans* homolog to human PPARγ, PPARα, and PPARδ, and that RNAi knockdown during development should reduce obesogenic potential of a coexisting teratogen exposure.

The following specific aims will be used to address the above hypothesis:

Specific Aim 1: Characterize the reprotoxic effects of chronic obesogen exposure in *C. elegans*. Parental *C. elegans* exposed to 0.1μM-100μM of putative obesogens for approximately 48 hours will be examined over four days during which the following data will be collected: eggs laid, resulting hatches, and embryonic percent viability. The
results of this study will be used in the selection of sub-embryotoxic doses for the obesogen screen in Specific Aim 2.

Specific Aim 2: Screen putative obesogens at sub-embryonic lethal doses for their effect on offspring lipid storage and energy balance. Offspring exposed in utero will be metabolically phenotyped as adults via the following techniques: Oil Red-O staining to address overall changes in lipid storage, pharyngeal pumping assay to address energy intake, and body thrashing assay to address energy expenditure.

Specific Aim 3: Ascertain and functionally assess putative C. elegans homologs to human PPARs (hPPARs). Bioinformatic analysis should reveal potential C. elegans nuclear hormone receptor homologs to human PPARγ, PPARα, and PPARδ, whose genes expression is repressed via RNAi, blocking changes in lipid storage induced by obesogenic teratogens, such as BPA. Metabolic phenotyping techniques used in Aim 2 in addition to a colorimetric coupled enzymatic triglyceride assay should assess the functional role of these NHRs in C. elegans lipid storage within the context of obesogens.

1.16 Significance and Impact

The results of this work should serve to highlight two salient facts concerning environmental health: First, chronic exposures to teratogens have the potential to adversely affect our reproductive ability. And second, chronic exposure to teratogens can alter the early development of offspring in utero, such that they are born with a metabolic foundation predisposed towards weight gain and obesity. Hopefully, the techniques presented here may be further applied to the assessment of
pharmaceuticals and constituents for consumer goods prior to their entry into the market.

1.17 References


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1.18 Tables and Figures

**Figure 1.1: An Illustrated Schematic for the Proposed Study.** *Caenorhabditis elegans* is used as an *in vivo* system to model teratogen exposures. These teratogens may be reprotoxic, inducing infertility or embryonic death. If viable offspring are produced, these teratogens have the potential to act as obesogens in the resultant offspring, increasing lipid storage. This increase in lipid storage may be due to positive changes in energy balance where offspring are consuming more calories than they are expending, and increased pro-lipogenic signaling activity of nuclear hormone receptors.
CHAPTER 2

REPRODUCTIVE TOXICITY OF SELECT DEVELOPMENTAL TERATOGENS

2.1 The Study of Teratogen Induced Reproductive Toxicity and Its Implications

Infertility may arise as a result of the toxic effects of environmental teratogens that function by interfering with reproductive function, fertilization, and embryonic viability as previously detailed in Chapter 1. Teratogens like BPA for example, promote aneuploidy which can reduce embryonic viability (Allard and Colaiácovo, 2010). Alternatively, teratogens like cigarette smoke, may function to deplete the germ cell pool, thereby reducing the number of these cells available for fertilization (Sobinoff et al., 2013). Lastly, others like triclosan have demonstrated embryotoxic effects that still warrant further mechanistic evaluation (Rodriguez and Sanchez, 2010). The goal of the current study is to address the reproductive toxicity of various, high-profile teratogens highlighted within the literature. We use a chronic exposure model in Caenorhabditis elegans to reveal changes in reproductive fitness induced by pervasive, environmental teratogens. To this end, we establish doses for future work designed to screen for obesogens, such that a sufficient number of surviving offspring is available for analysis.

While this study is designed to select sub-embryotoxic doses for the future obesogen screen, the results presented here have implications for the study of infertility as well. Infertility is recognized as a global issue by the World Health Organization, and affected 48.5 million couples during 2010 (Mascarenhas et al., 2012). However, only
roughly half of affected couples seek treatment (Boivin, 2007). This may be due to clinic availability and cost (Inhorn and Patrizio, 2015). Many women who suffer from infertility in low-income countries have little access to assisted reproduction treatments (ART), as the number of clinics in Sub-Saharan Africa, for example, pale in comparison to those of Japan (Inhorn and Patrizio, 2015). The cost of ART is also a driving factor for couples to engage in ‘catastrophic expenditure’, where more than 40% of the yearly non-food budget is on one commodity (Dyer and Patel, 2012). Inhorn and Patrizio postulate to address the problem of infertility, we must not only heighten awareness of the issue but also find and address its preventable causes (Inhorn and Patrizio, 2015).

The current study uses chronic, low-dose teratogen exposure in vivo in C. elegans to reveal changes in reproductive development, fertility, and embryo viability that may benefit the study of infertility. However, these reproductive toxicity studies are also necessary for dose selection in work designed to properly address the Fetal Origins of Health and Disease Hypothesis. In order to perform the obesogen screen detailed in Chapter 3, it was necessary to select a dose that does not delay the onset of reproductive maturity and does not significantly reduce the number of offspring produced. The short, characterized lifespan of C. elegans facilitates tracking these qualities as a function of egg-laying and larval hatching over the first several days of its adult life.

2.2 Hypothesis

Chronic exposure to teratogens in C. elegans will have adverse effects on fertility and reproduction as revealed via changes in egg-laying, hatching, and embryonic percent viability throughout the reproductive lifespan.
2.3 Methods

2.3.1 *C. elegans* Strain and Colony Maintenance

The *Caenorhabditis elegans* strain being used is the N2 Bristol strain. *C. elegans* were housed in a low-temperature Heratherm incubator (Thermo Scientific, Waltham, MA) at 25°C on either 60mm or 100mm petri dish plates containing NGM (nematode growth media). The NGM used is NGM-lite (N1005, US Biologics, Salem, MA) prepared according to manufacturer’s instructions: 25g per 1 liter of ultrapure water mixed thoroughly until solubilized, autoclaved at 121°C for 15 minutes. Live *Escherichia coli* bacteria (strain OP50), was used as a food source for the *C. elegans*. The bacterial feeding stocks were grown in LB broth at 37°C and 220 RPM for 16-18 hours overnight in incubator bacterial shaker (Excella E24 Incubator Shaker Series, New Brunswick Scientific, NJ, USA). After incubation, the optical density was measured and adjusted with the same batch of LB broth to obtain a final optical density of 1.0. Feeding stock was added to the NGM plates as a 50μL drop of re-suspended culture. This drop was spread across the entire plate and incubated at 25°C for approximately 18 hours overnight. OP50 was chosen as the feeding strain because it is a uracil auxotroph. This characteristic results in a slower growth than other strains of *E. coli* which ultimately aids in preventing the formation of a thick bacterial lawn. Minimization of the lawn was advantageous as it allowed improved visualization and counting of embryos and hatched *C. elegans*. The following day these plates were seeded with larval *C. elegans*. 
2.3.2 Developmental Age-synchronization

In order to synchronize the age of the parental generation of *C. elegans*, embryos were harvested from three to four mixed developmental stage plates of nematodes. First, nematodes were collected and rinsed from each plate with M9 buffer (3g KH$_2$PO$_4$, 6g Na$_2$HPO$_4$ & 5g NaCl per 1L ultrapure H$_2$O) into a 15 mL conical vial. The vial was centrifuged at 1000 RPM for 1 min and excess M9 was aspirated off to approximately the 0.5mL mark on the conical vial. Fresh M9 was added again, filling the vial. Two more cycles of centrifugation and aspiration were performed. Secondly, an alkaline bleach solution was prepared by first diluting 3.8mL of 8.25% bleach (Clorox brand) with 6.2mL ultrapure water, yielding a final concentration of 3.135% bleach. To this, 0.2g of sodium hydroxide was added and dissolved by vortexer. Four milliliters of this alkaline bleach solution was added to the 0.5mL of nematode sample in M9 remaining in the conical vial. The contents were immediately mixed by vortexer for 20 s, manually shaken for 2 min, again mixed by vortexer for 20 s, after which 11 mL of M9 buffer was quickly added. The alkaline bleach solution functions to dissolve the cuticle of the nematode. The embryos however are covered in a thick chitinous shell, which makes them relatively resistant to this short exposure to bleach. The conical vial was then centrifuged at 1000 RPM for 1 min and the diluted bleach solution was aspirated off to approximately the 0.5mL mark on the conical vial. After this, 14.5 mL of M9 was used to perform 3 washes on the remaining pelleted eggs, with a centrifugation and aspiration to approximately 0.5 mL between each wash. After the last wash, the volume in the conical vial was brought up to four milliliters with M9 buffer. With the conical vial firmly capped, it was placed on a three-dimensional shaking motion rotator (GyroTwister
GX-1000, LabNet International, Edison, NJ, USA) for 18-20 hours overnight at 15 rotations/min to allow the released eggs to hatch. In the absence of any food source, these hatched larvae arrest at the first larval stage (L1).

2.3.3 Chemicals

Nine of the ten teratogens were purchased from Sigma Aldrich (St. Louis, MO, USA). These include tributyltin-chloride \( \text{C}_{12}\text{H}_{27}\text{ClSn} \), cadmium-chloride \( \text{CdCl}_2 \) anhydrous, benzo-\( \alpha \)-pyrene \( \text{C}_{20}\text{H}_{12} \), nicotine \( \text{C}_{10}\text{H}_{14}\text{N}_2 \), arsenic (III) oxide \( \text{As}_2\text{O}_3 \), triclosan \( \text{PHR1338} \), and fenthion \( \text{C}_{10}\text{H}_{15}\text{O}_3\text{PS}_2 \). The CSE (cigarette smoke extract) was prepared as a distillate from the Kentucky Reference cigarette 3R4F that is adjusted by the manufacturer to a final concentration of 40 mg/ml of TPM (total particulate matter) in DMSO, (Murty Pharmaceuticals (Lexington, KY, USA). All teratogens were dissolved in 100% DMSO except arsenic(III)oxide, which was found to be relatively insoluble in DMSO. Arsenic (III) oxide was dissolved in ultrapure water in a 15mL conical vial immersed in a water bath at 35° C. For each dose of arsenic (III) oxide tested, an equivalent amount of DMSO added to the NGM agar to bring the final concentration to 0.01% DMSO. Cigarette smoke extract was diluted in DMSO according manufacturer’s specifications for the 3R4F Reference Cigarette, provided by the University of Kentucky Center for Tobacco Reference Products (Lexington, KY, USA). CSE was prepared at a concentration equivalent to those tested for nicotine.

400µL of each teratogen DMSO stock was added directly to 39.6mL of NGM lite agar and was mixed by pipetting the solution with a serological pipette or vortexing on a
low setting in order minimize generating bubbles. This generated 0, 0.1, 0.5, 1.0, 5.0, 10, 50, and 100 μM dilutions of each teratogen in 0.01% DMSO in NGM agar. 400μL of 100% DMSO was added to 39.6mL of NGM to generate 0.01% DMSO vehicle control (0 μM) plates. Untreated control plates were made from the same batch of NGM. Approximately 6mL of each dilution in NGM was poured into each 60mm petri dish for each exposure group.

Because these teratogens pose a threat to human health and the environment, precautions were taken under the recommendation of the University of South Florida's Environmental Health and Safety Department to minimize and properly dispose of all waste produced in the generation of these dilutions.

2.3.4 Exposure Design

On the first day of the experiment, approximately 400 adult nematodes were rinsed from six, mixed developmental stage nematode plates (60 mm). These worms were age-synchronized with alkaline bleach and left to hatch as described above. On this day the experimental NGM plates were seeded with 50μL of OP50 feeding stock, let to dry in a closed hood, and incubated for approximately 16-18 hours overnight at 25°C. All control and experimental NGM plates were coded. All experiments were performed and data were collected double blind with the observer unaware to experimental conditions. Data was decoded upon collection and submission to the coder. On the second day, the vial of L1 larvae was incubated at 4°C for 5 minutes, centrifuged for 2 minutes at 1000 RPM, and excess M9 buffer was aspirated to approximately the 0.5 mL mark. The L1 pellet was resuspended by gently tapping the bottom of the vial or vortexing on the lowest setting. Three 10μL drops of the resuspended worm sample
were pipetted onto the lid of an empty petri dish. The number of L1 nematodes were counted in each drop and averaged to estimate the number of L1 larvae per microliter in the sample suspension. 10-50 µL M9 buffer containing at least 50 L1 larvae in total was placed onto each plate and allowed to dry under a closed hood. Dry plates were sealed with parafilm and placed into the low-temperature incubator for approximately 48 hours at 25°C. With respect to the experiments evaluating triclosan exclusively, 10 µL of 10X OP50 feeding stock needed to be added to each plate after the first 24 hours of exposure as the agent limited the growth of our feeding strain.

24-well plates were used to assess egg-laying and hatching over 4 days after the initial 48 hr exposure. One 24-well plates per group for each replicate was made with 0.5mL of untreated NGM per well (Figure 2.1). Each row in the 24-well plate corresponded to a day of analysis. One plate was used per group per replicate. On the day before parental worm transfer to the 24-well plate, a bacterial lawn was made with 10 µL of OP50 feeding stock in each well of the appropriate row. The lawn was spread by making a miniature version of a bacterial spreader from a Pasteur pipette with the flame from a Bunsen burner. 24-well plates were incubated for 16-18 hr at 25°C overnight. After 48 hours of teratogen or control exposure one parental (P0) C. elegans was moved to each well of row A of the respective 24-well plate for its group. The P0 nematode was moved to the row below approximately every 24 hours, after which the number of eggs laid the day prior was counted. Hatched larvae were counted on the subsequent day. Counts of eggs and hatched larvae per P0 nematode were made on each day for 4 days. Embryonic percent viability was calculated as the percent of eggs
that had successfully hatched within the previous 24 hours. Upon each transfer notations were also made of dead, accidently killed, missing, or escaped P0 C. elegans.

2.3.5 Statistical Analysis

The number of eggs laid and the number of hatched larvae was recorded for each parental C. elegans over the four days post-exposure. The number of hatched larvae was divided by the number of eggs laid, then multiplied to 100 to calculate the embryonic % viability. Data were reviewed prior to analysis for entry errors which included the following: wrong numbers entered into cells (typos), right numbers entered into wrong cells, eggs and hatch discordance. Entry errors were corrected and the number of errors recorded to obtain the percent error rate which was 0.57% for all instances of eggs and hatches observed from parental C. elegans over the four days post-exposure. In the majority of treatment groups and experiments, maximal egg-laying was noted on the first day post-exposure. Since later projects require maximal offspring yields, further analyses of egg-laying, hatching, and embryonic viability were focused on data from this day. Statistical Package for the Social Sciences (IBM SPSS Version 23) was used to perform descriptive and inferential statistical analyses, and generate corresponding figures. Data were analyzed via one-way ANOVAs followed by LSD and Dunnett’s C post-hoc analyses (for data that did and did not adhere to the assumed homogeneity of variance, respectively). GraphPad Prism (GraphPad Software Inc., Version 6.0h) was used to transform and normalize data in order to calculate effective concentrations that reduced day 1 post-exposure egg-laying (EC50) and embryonic lethality (LD50), using a least-squares fitted nonlinear regression model where applicable. All control and experimental groups were measured in three-to-five
replicates with at least 18-36 P0 nematodes for most groups with a total N = 1692 P0 nematodes for the whole study.

2.4 Results

2.4.1 Bisphenol-A Affects Egg-laying and Hatching

Chronic parental (P0) exposure to BPA affected egg-laying and hatching, but not embryonic viability in *C. elegans*. Parental nematodes in all treatment groups laid the most eggs and had the corresponding highest number of hatched larvae on the first day post-exposure (Figure 2.2). Regarding day 1 post-exposure egg-laying, box-plots of egg-laying and hatching reveal a seemingly non-monotonic, U-shaped dose-response curve (Figure 2.3). P0 nematodes exposed to 50.0μM BPA laid the highest significant overall mean eggs (84.50 ± 7.55 SEM) on day 1 compared to vehicle control (66.06 ± 6.08 SEM), (p<0.05). P0 nematodes exposed to 0.5μM BPA laid the lowest significant overall mean eggs (41.41 ± 5.87 SEM) on day 1 compared to vehicle control (ANOVA F(7, 246)=4.856, p<0.01). Regarding day 1 post-exposure hatching, P0 nematodes exposed to 100.0μM BPA had the highest significant overall mean hatched larvae (55.23 ± 6.47 SEM) on day 1 compared to vehicle control (36.47 ± 4.95 SEM), (ANOVA F(7, 246)=4.287, p<0.01). Overall, there were no significant differences in embryonic viability on day 1 post-exposure from P0 exposed chronically to BPA when compared to vehicle control.

2.4.2 Diethylstilbestrol Affects Egg-laying

Chronic parental (P0) exposure to DES significantly affected egg-laying, but not hatching or embryonic viability in *C. elegans*. Parental nematodes in all treatment groups laid the most eggs and had the corresponding highest number of hatched larvae
on the first day post-exposure (Figure 2.5). Regarding day 1 post-exposure egg-laying, box-plots of egg-laying and hatching reveal a relatively steady levels of egg-laying and larval hatches at doses lower than 50.0μM (Figure 2.3). P0 nematodes exposed to 50.0μM and 100.0μM DES laid the lowest significant overall mean eggs (65.94 ± 4.59 and 47.88 ± 3.54 SEM, respectively) on day 1 compared to vehicle (86.40 ± 4.46 SEM), (ANOVA F(7, 146)=7.667. p<0.001). Regarding day 1 post-exposure hatching, P0 nematodes exposed to 100.0μM DES had the lowest significant overall mean hatched larvae (34.69 ± 4.38 SEM) on day 1 compared to vehicle (62.71 ± 4.52 SEM) (ANOVA F(7, 146)=2.391, p<0.05). Overall, there were no overall significant differences in embryonic viability on day 1 post-exposure from P0 exposed chronically to DES when compared to vehicle controls.

2.4.3 Triclosan Affects Egg-laying and Hatching

Chronic parental (P0) exposure to triclosan affected egg-laying and hatching, but not embryonic viability in C. elegans. Parental nematodes in most treatment groups laid the most eggs and had the corresponding highest number of hatched larvae on the first day post-exposure. Only P0 nematodes exposed to 5.0μM and 10.0μM laid slightly more eggs on day 2 post-exposure, although this phenomenon was not noted for the hatched larvae data (Figure 2.5). Regarding day 1 post-exposure egg-laying, box-plots of egg-laying and hatching reveal downward trend of egg-laying and larval hatches as the dose of triclosan increases (Figure 2.8). P0 nematodes exposed to doses of 5.0μM and 10μM triclosan laid significantly lower overall mean eggs (34.29 ± 5.75 and 34.69 ± 5.35 SEM, respectively) on day 1 compared to vehicle (74.51 ± 4.08 SEM), (ANOVA F(7,150)=33.905. p<0.001). P0 nematodes exposed to doses of 50.0μM and 100.0μM
laid no eggs on day 1 post-exposure, and all but one in the 100.0μM group had died by day 2 post-exposure (Figure 2.8). A nonlinear regression of normalized egg-laying data plotted against log [μM Triclosan] shows a 50% reduction in egg-laying IC50=4.262μM (95% CI [2.962, 6.748], R²=0.4727) (Figure 2.11). Regarding day 1 post-exposure hatching, P0 nematodes exposed to 5.0μM DES had the lowest overall significant mean hatched larvae (23.76 ± 3.99 SEM) on day 1 compared to vehicle (46.49 ± 4.74 SEM) (ANOVA F(7,150)=13.395, p<0.001). Overall, there were no overall significant differences in embryonic viability on day 1 post-exposure from P0 exposed chronically to triclosan doses less than 10μM when compared to vehicle controls.

2.4.4 Tributyltin Affects Egg-laying, Hatching, and Embryonic Viability

Chronic parental (P0) exposure to TBT affected egg-laying, hatching, and embryonic viability in C. elegans. Parental nematodes in the 0.1μM exposure group laid the most eggs and had the corresponding highest number of hatched larvae on the first day post-exposure (Figure 2.11). In all other TBT exposure groups, mean eggs laid increased from day 1 to day 2 post-exposure, with the most dramatic results seen in P0 nematodes from the 5.0μM and 10.0μM TBT exposure groups. Focusing on day 1 post-exposure specifically, box-plots of egg-laying and hatching reveal a dose-dependent decrease in the number of eggs laid and corresponding hatches (Figure 2.12). No eggs or hatches were noted on day 1 post exposure in the 5.0μM and 10.0μM TBT exposure groups. P0 nematodes exposed to 0.5μM and 1.0μM TBT laid the lowest significant overall mean eggs (49.22 ± 6.74 and 26.22 ± 6.35 SEM, respectively) on day 1 compared to vehicle (82.18 ± 4.46 SEM), (ANOVA F(5, 117)=64.799, p<0.01). A nonlinear regression of normalized egg-laying data plotted against log [μM TBT] shows
a 50% reduction in egg-laying IC50=0.6402μM (95% CI [0.5326, 0.7697], R²=0.7311) (Figure 2.11). Regarding hatching, P0 nematodes exposed to 0.5μM and 1.0μM TBT had the lowest overall mean hatched larvae (22.56 ± 4.20 and 16.00 ± 5.79 SEM) on day 1 compared to vehicle, however this statistic only approached significance (ANOVA F(5, 117)=29.412, p<.001). Regarding embryonic percent viability, offspring from P0 nematodes exposed to 0.5μM TBT had the lowest overall significant mean percent viability on day 1 post exposure (40.80% ± 5.25 SEM) compared to vehicle controls (66.62% ± 3.56 SEM) (ANOVA F(3,82)=5.828, p<0.01). Doses of 5.0μM and 10.0μM TBT appeared to delay P0 growth and reproductive ability, while doses of 50.0μM and 100.0μM TBT were P0 larval lethal. For a detailed analysis of these particular findings on delayed development please refer to the undergraduate honor’s thesis of Emily McIntyre (McIntyre, 2015).

2.4.5 Fenthion Affects Egg-laying and Hatching

Chronic parental (P0) exposure to fenthion affected egg-laying and hatching, but not embryonic viability in C. elegans. Parental nematodes in the 0.1μM-5.0μM treatment groups laid the most eggs and had the corresponding highest number of hatched larvae on the first day post-exposure. P0 nematodes exposed to 10.0μM-100.0μM treatment groups laid more eggs on day 2 post-exposure (Figure 2.5). Regarding day 1 post-exposure egg-laying, box-plots of egg-laying and hatching reveal a dose-dependent downward trend of egg-laying and larval hatches as the dose of fenthion increases (Figure 2.8). P0 nematodes exposed to doses of fenthion at 5.0μM and above laid significantly lower overall mean eggs (49.24 ± 5.87 SEM and fewer) on day 1 compared to vehicle (66.36 ± 3.58 SEM), (ANOVA F(7, 152)=8.163. p<0.001). A
linear regression of normalized egg-laying data plotted against log [μM Fenthion] shows a 50% reduction in egg-laying IC50=3.0608 μM ($R^2=0.8581$) (Figure 2.1). Regarding day 1 post-exposure hatching, P0 nematodes exposed to doses of fenthion at 5.0μM and above had the lower overall significant mean hatched larvae (29.82 ± 6.24 SEM) on day 1 compared to vehicle (45.22 ± 4.37 SEM) (ANOVA $F(7, 152)=3.695$ $p<0.01$). Overall, there were no overall significant differences in embryonic viability on day 1 post-exposure from P0 exposed chronically to fenthion when compared to vehicle controls.

2.4.6 Cigarette Smoke Extract Affects Hatching and Embryonic Viability

Chronic parental (P0) exposure to CSE affected hatching and embryonic percent viability, but not egg-laying in *C. elegans*. Parental nematodes in all treatment groups laid the most eggs and had the corresponding highest number of hatched larvae on the first day post-exposure (Figure 2.17). Regarding day 1 post-exposure egg-laying and hatching, box-plots of these data reveal no apparent trend (Figure 2.18). Indeed, no significant differences in egg-laying on day 1 post-exposure were noted in any of the groups upon post-hoc analysis. Regarding day 1 post-exposure hatching, P0 nematodes exposed to doses of CSE doses at 50.0μM and 100μM had the highest overall significant mean hatched larvae (45.06 ± 5.79 and 51.41 ± 7.02 SEM, respectively) on day 1 compared to vehicle (28.23 ± 4.27 SEM) (ANOVA $F(7, 152)=2.425$, $p<0.05$). Similarly, offspring from these two exposure groups also exhibited the highest significant overall mean embryonic percent viability (60.24 ± 6.90% and 64.24 ± 6.83% SEM, respectively) when compared to vehicle control (37.41 ± 5.05% SEM) (ANOVA $F(7,152)=3.258$, $p<0.005$).
2.4.7 Nicotine Does Not Affect Egg-laying, Hatching, or Embryonic Viability

Chronic parental (P0) exposure to nicotine had no effect on egg-laying or hatching in *C. elegans*, (ANOVA F(7, 152)=0.505, p=0.830), (ANOVA F(7, 152)=1.047, p=0.401). Parental nematodes in all treatment groups laid the most eggs and had the corresponding highest number of hatched larvae on the first day post-exposure (Figure 2.20). Regarding day 1 post-exposure egg-laying and hatching, box-plots of egg-laying and hatching reveal no apparent trend (Figure 2.21). Indeed, no significant differences in egg-laying or hatching on day 1 post-exposure were noted in any of the groups upon post-hoc analysis. No significant effects were found in embryonic viability in comparison to the vehicle control group, although offspring from the 5.0μM exposure group had the lowest embryonic viability that approached statistical significance (p=0.067). Offspring embryonic viability was highest, approaching statistical significance in the 50.0μM exposure group (Figure 2.22, p=0.065).

2.4.8 Benzo-α-pyrene Affects Egg-laying and Hatching

Chronic parental (P0) exposure to BAP affected egg-laying and hatching, but not embryonic viability in *C. elegans*. Parental nematodes in the 0.1μM-1.0μM and 50.0μM treatment groups laid the most eggs and had the corresponding highest number of hatched larvae on the first day post-exposure. P0 nematodes exposed to 5.0μM, 10.0μM, and 100.0μM treatment groups laid more eggs on day 2 post-exposure (Figure 2.23). Regarding day 1 post-exposure egg-laying, box-plots of egg-laying and hatching reveal a slight downward trend of egg-laying and larval hatches as the dose of BAP increases (Figure 2.24). P0 nematodes exposed to doses of BAP at 5.0μM and above laid significantly lower mean eggs (34.94 ± 4.01 SEM and fewer) on day 1 compared to
vehicle ($63.72 \pm 4.76$ SEM), (ANOVA $F(7, \ 150)=13.12. \ p<0.001$). A nonlinear regression of normalized egg-laying data plotted against log [$\mu$M BAP] shows a 50% reduction in egg-laying IC50=1.691 $\mu$M (95% CI [0.9672, 2.958], $R^2=0.3798$) (Figure 2.26). Regarding day 1 post-exposure hatching, P0 nematodes exposed to doses of BAP at 5.0μM, 10.0μM, and 100.0μM had the lowest significant mean hatched larvae (21.18 $\pm$ 3.46 SEM and fewer) on day 1 compared to vehicle (43.64 $\pm$ 4.82 SEM) (ANOVA $F(7, \ 150)=7.310, \ p<0.001$). There were no significant differences in embryonic viability on day 1 post-exposure from P0 exposed to BAP when compared to vehicle.

2.4.9 Cadmium Affects Egg-laying, Hatching, and Embryonic Viability

Chronic parental (P0) exposure to cadmium affected egg-laying and hatching, but not embryonic viability in *C. elegans*. Parental nematodes in most treatment groups laid the most eggs and had the corresponding highest number of hatched larvae on the first day post-exposure. Only P0 nematodes exposed to 5.0μM and 10.0μM laid slightly more eggs on day 2 post-exposure (Figure 2.26). Regarding day 1 post-exposure egg-laying, box-plots reveal dose-dependent downward trend of egg-laying as the dose of cadmium increases from 0.5μM (Figure 2.27). P0 nematodes exposed to doses of 5.0μM and 10μM cadmium laid significantly lower overall mean eggs ($17.00 \pm 3.18$ and $4.67 \pm 1.38$ SEM, respectively) on day 1 compared to vehicle ($61.95 \pm 4.79$ SEM), (ANOVA $F(7, \ 186)=30.174. \ p<0.001$). P0 nematodes exposed to doses of 50.0μM and 100.0μM laid no eggs on day 1 post-exposure. A nonlinear regression of normalized egg-laying data plotted against log[$\mu$M Cd] shows a 50% reduction in egg-laying IC50=1.2.086 $\mu$M (95% CI [1.451, 2.998], $R^2=0.4825$) (Figure 2.27). All but 5 P0 in the 50.0μM group and 1 P0 in the 100.0μM group had died by day 4 post-exposure (Figure
2.26). Regarding day 1 post-exposure hatching, boxplots reveal a dramatic reduction in all cadmium treated groups, although only those in the 5.0μM and 10.0μM reached statistical significance (4.58 ± 6.47 and 1.0 ± 2.62 SEM, respectively) on day 1 compared to vehicle (26.38 ± 3.78 SEM) (ANOVA F(7, 186)=9.465, p<0.001). Regarding embryonic viability, boxplots reveal a reduction in most of the cadmium treatment groups. Indeed, all doses of cadmium 10.0μM and below, with the exception of 0.5μM, significantly reduce embryonic viability (21.99 ± 4.99% SEM and less) compared to vehicle control (38.13 ± 4.82% SEM) (ANOVA F(5, 129)=2.367, p<0.05).

2.4.10 Arsenic(III)oxide Affects Egg-laying, Hatching, and Embryonic Viability

Chronic P0 exposure to arsenic(III)oxide affected egg-laying, hatching, and embryonic viability in C. elegans. P0 nematodes in all treatment groups laid the most eggs and had the highest number of hatched larvae on the first day post-exposure (Figure 2.29). Box-plots reveal a relatively steady mean number of eggs on day 1 post-exposure laid across all treatment groups less than 50.0μM (Figure 2.30). P0 nematodes exposed to 50.0μM and 100.0μM arsenic(III)oxide laid significantly lower mean eggs (39.39 ± 3.91 and 40.44 ± 5.01 SEM, respectively) on day 1 compared to vehicle (78.47 ± 3.85 SEM), (ANOVA F(7, 152)=16.309. p<0.001). P0 nematodes exposed to 0.10μM arsenic(III)oxide laid the highest significant mean eggs (99.78 ± 6.43 SEM). Nonlinear regression of normalized egg-laying data against log[μM As(III)O] shows a 50% reduction in egg-laying IC50=16.07 μM (95% CI [7.440, 34.7], R²=0.1960) (Figure 2. A). P0 nematodes exposed to 50.0μM and 100.0μM arsenic(III)oxide had significantly fewer mean hatches (19.22 ± 3.77 and 26.00 ± 3.75 SEM, respectively) on day 1 compared to vehicle (56.88 ± 5.34 SEM) (ANOVA F(7, 152)=13.384, p<0.001).
Boxplots reveal similar trends in embryonic viability to those seen in egg-laying and hatching analyses. Offspring of P0 nematodes exposed to 50.0μM arsenic(III)oxide had significantly reduced embryonic viability (44.84 ± 7.00% SEM) compared to vehicle control (70.17 ± 5.27% SEM) (ANOVA F(7, 149)=5.474, p<0.001). Nonlinear regression of normalized embryonic lethality data against log[μM As(III)O] shows an LD50=13.41 μM (95% CI [8.694, 20.69], R²=0.4245) (Figure 2.32B). Offspring of P0 nematodes exposed to 0.50μM arsenic(III)oxide had the highest significant mean embryonic viability (91.27 ± 3.35% SEM) compared to vehicle.

2.5 Discussion

At least one treatment group in all of the tested compounds except nicotine significantly affected average egg-laying, hatching, or embryonic viability on day 1 post-exposure (Table 2.1). Five of the ten compounds when tested at doses of 1.0μM-5.0μM or above shifted the day of maximal P0 egg-laying (Table 1). Some of these exposures at doses of 5.0μM and above appeared to reduce P0 size. The reduction in size is reflective of a developmental delay, where the gonads may not yet be fully functional for purposes of self-fertilization. This phenomenon is covered in greater detail in the Honor’s Undergraduate Thesis of Emily McIntyre (McIntyre, 2015).

Only three of the six compounds (TBT, Cadmium, As(III)O) that reduced embryonic viability on day 1 post-exposure at higher dosages concomitantly decreased the number of eggs laid. For the remaining four compounds that reduced egg-laying (BPA, DES, Triclosan, Fenthion), significant differences in embryonic viability were not noted. These data indicate that as P0 were exposed to increasingly reprotoxic doses of the later compounds, total number of eggs produced was reduced while these embryos
maintained their overall viability. We can infer two potential reasons for this phenomenon. The first explanation is that total offspring yields for the P0 were being sacrificed for increased likelihood of offspring survival. A second likely explanation is that the exposure itself reduced the germ-cell pool of the exposed P0. Sobinoff et al. reported in 2013 that cigarette smoke in the C57BL/6 mouse induced apoptosis predominantly in secondary follicles. This caused a significant reduction in the pool of primordial and primary follicles as they were hastened towards replacement of secondary follicles (Sobinoff et al., 2013). To examine if analogous changes are occurring in C. elegans as a result of our exposures, future work may be best directed at gonadal analysis on day 1 post-exposure. In this technique, individual arms of the worm gonad are carefully dissected out with 28- or 30-gauge needles. These gonads may be imaged with the stain or antibody of interest, providing information on the number of developing or apoptotic germ cells as they progress through meiosis. This technique has been successfully used to demonstrate the reprotoxic effects of BPA and several pesticides in C. elegans (Allard and Coláicovo, 2010; Parodi, Damoiseaux, and Allard, 2015). In one such example from 2010, Allard and Coláicovo demonstrated that BPA exposure from 100, 500, and 1 mM in C. elegans reduced the number of eggs laid at the highest dose and increased embryonic lethality in a dose-dependent fashion. DAPI-stained whole gonads from BPA-exposed worms were noticeably smaller than those of vehicle control worms. Notably, their dose response tested a range of 10 fold higher doses, suggesting that BPA exposure may need to be tested at broader range up to 1 mM exposure to detect changes. The localization of RAD-51, a protein involved in double-strand break repair typically during the mid-pachytene stage of meiosis, was
also examined in four gonads each from vehicle and BPA exposed nematodes. In the BPA-exposed gonads, RAD-51 was noted at increasingly later stages of pachytene progression. Indicating a shift in the double-strand break repair process, this effect significantly increased apoptosis of germ cells in the late pachytene stages as indicated by acridine orange staining. The transparent nature of the *C. elegans* gonad lent itself well to the staining and analysis of meiotic abnormalities that explained the observed reduction in eggs and live offspring (Allard and Colácico, 2010). TUNEL (TdT-mediated dUTP Nick End Labeling) staining and corpse visualization via DIC microscopy may also be used to detect apoptotic cells in the *C. elegans* gonad (Gartner, Boag, and Blackwell, 2008). Potential implicated pathways may be assessed by measuring the expression of genes that are known to be protective against apoptosis in the *C. elegans* germline. Several of these, known to regulate the processes of transcription or translation, have RNAi knockdown reproductive phenotypes that similarly reduce embryonic viability or the number of live offspring (Gartner, Boag, and Blackwell, 2008).

### 2.5.1 Specific Reprotoxic Effects of Estrogen Mimics

Regarding estrogen mimics, both BPA and DES reduced the number of eggs laid, although the doses at which they exerted this effect differed. DES had no significant effects at lower doses, however at one of the highest doses of 50μM, it significantly decreased the number of eggs laid. Conversely, a lower dose of 0.5μM BPA exposure were shown to significantly reduce the number of eggs laid while a higher dose of 50.0μM was shown to significantly increase the number of eggs laid. DES is known to be several orders of magnitude more potent of an estrogen agonist...
than BPA (Dodds, 1938). Because of this, we would expect lower doses of DES to exert similar reprotoxic effects to those seen resulting from BPA tested at higher doses. This however was not the case, suggesting that BPA and DES while both estrogen mimics may also have other target molecules or modulate differing downstream pathways responsible for the observed reprotoxic effects.

Additionally, our results show significantly increased egg-laying at the highest dose of BPA administered with no significant difference in embryonic viability compared to vehicle control. These results are in contrast with the previously mentioned, prior work published by Allard and Coláicovo in 2010. This may be attributed first to differences in doses tested. Allard and Coláicovo’s lowest tested dose of BPA, 100μM, was our highest dose tested. In our study, we evaluated doses from 0.1μM to 100μM of BPA in NGM, effectively recapitulating the chronic low-dose exposure model for C. elegans that was initially presented in early work using mice by vom Saal in 1998. In the work by Allard and Coláicovo, they showed that there were still sufficient numbers of eggs laid from 100μM BPA treated parents compared to their vehicle control group. They did however show that these 100μM BPA exposed eggs were only about 60% as viable as control-treated eggs, whereas we noted a comparative increase in our embryonic percent viability that approached significance (p=0.057). This discrepancy may be due to their choice of vehicle (0.1% Ethanol). To justify this first, a small initial study by our lab revealed that across varying doses of chronic parental exposure to DMSO (0.01%, 0.1%, and 1.0% in NGM) there were no observable differences in embryonic viability. However chronic exposure to two doses of ethanol (0.1% and 1.0% in NGM) revealed that 1.0% ethanol was especially repro-toxic compared to the other 5
groups. Because of these results, we chose to use DMSO as our vehicle since it was the most consistent across doses and slightly less embryotoxic than 0.1% EtOH. Secondly, in the establishment of our assays for these protocols we realized that the developing embryos of parental nematodes who had chronic exposure to reprotoxic agents (BPA in particular) were especially susceptible to the toxic effects of the alkaline bleach age synchronization procedure. Embryos are encased in the chitinous shell of the egg and are relatively impervious to this acute, transient exposure. However, we noticed in BPA-exposed offspring drastically increased the amounts of embryonic lethality with virtually no hatched larvae after this procedure. After several trials, we concluded that the in utero exposure to the reprotoxic agent combined with the bleach-exposure for age synchronization was too harsh a condition for the embryos to withstand. We switched to an egg-laying based protocol and noticed consistently improved results (D. Flaherty, personal communication; 2013). Because we have noticed that (1) ethanol is itself embryotoxic, and (2) embryos are unable to withstand combined embryotoxic insults; it is possible that the findings in the 100μM treated group of Allard and Colaiácovo’s work are due to synergistic effects of BPA and ethanol, rather than those of BPA alone.

Our results pertaining to DES exposure however appear to be in-line with prior work from Goldstein and Aun in 1993. In an attempt to isolate DES-surviving mutants, they noticed the threshold for cytotoxicity was 100μM. Furthermore, they noted that those worms who did survive ultimately produced fewer eggs (Goldstein and Aun, personal communication; 2016). Indeed, at doses of 50μM and 100μM DES, we noticed a significant reduction in overall mean eggs laid on day 1 post-exposure, with a
concomitant significant reduction in the number of hatches from the 100μM DES-treated group. However only parental worms in the 50μM treatment group showed a slight, non-significant increase in overall mortality across the four days of study (data not shown).

Taken together, these data highlight that even different agents within the same chemical class may have opposing effects on the developing germline and offspring depending on the dose at which they are administered. While DES causes reduced fertility at the 100μM dose, BPA causes increased fertility at the 100μM dose and reduced fertility at the low, 0.5μM dose. DES was chosen as a pharmaceutical specifically for its estrogen-receptor agonizing activity (Dodds, 1938). However BPA has been shown to bind a number of other nuclear receptors, including the androgen receptor, steroid and xenobiotic receptor, and peroxisome proliferator activated receptor, and thyroid hormone receptor (Li et al., 2015). Our results in conjunction with the highlighted literature suggest that BPA and DES may be acting through different mechanisms to exert their effects on reproductive fitness, despite both being estrogen mimics.

2.5.2 Specific Reprotoxic Effects of Biocides

Regarding biocides, all three tested had significant inhibitory effects on egg-laying and shifted the day of maximal egg-laying for at least one of the doses tested. While several articles have examined TBT using C. elegans as a model system (Wang et al., 2012; Wang et al., 2014; Cheng et al., 2014), and one study has similarly examined triclosan (Iaokuchi et al., 2014), to our knowledge this study is the first to address the reprotoxic effects of fenthion in C. elegans.
Triclosan significantly reduced the number of eggs laid in the 5.0μM and 10.0μM treatment groups, which resulted in a significant decrease in the number of hatches for the 5.0μM group only. The day of maximal egg-laying was also shifted to day 2 post-exposure for parent *C. elegans* in these two treatment groups, suggesting a developmental delay. No eggs were laid in the 50.0μM and 100.0μM treatment groups. Nonlinear regression modeling of this data revealed an IC50_{Egg-laying}= 4.262 (95% CI 2.962, 6.748; R²=0.4727). While the CI and R² values for this analysis suggest a poor fit, the shift in day of maximal egg-laying is in line with the only other prior study designed to address this effect. Iaokuchi et al. found in 2012 that the lowest observed effective chronically administered concentration to delay maturation in *C. elegans* at 20°C was 10.81μM triclosan (Iaokuchi et al., 2012). However, they did not observe significant decreases in egg-laying in response to their highest dose tested (approximately 21.59μM triclosan). Because of this discrepancy, further work is warranted to better assess the potential reproductive toxicity of triclosan using *C. elegans* and better pinpoint the effective reprotoxic concentrations.

TBT significantly reduced the number of day 1 post-exposure eggs laid and the number of hatched larvae in a dose-dependent fashion for all doses tested except 0.1μM. Nonlinear regression modeling of this data revealed an IC50_{Egg-laying}= 0.6402μM (95% CI 0.5326, 0.7697; R²=0.7311). Correspondingly, eggs laid by parents in the 0.5μM TBT exposure group also showed significantly reduced embryonic percent viability. The inability to observe this effect in the 1.0μM treatment group may be due to its increased variability. Parental *C. elegans* exposed to 5.0μM and 10.0μM TBT did not lay any eggs on the first day post-exposure and were noticeably smaller in size. The
nematodes in these two exposure groups however did recover by the third day post-exposure, laying maximal eggs. The shift in the day of maximal egg-laying indicated a developmental delay. The later results explain why prior work evaluating the mechanisms behind TBT-mediated reproductive toxicity have not examined doses higher than 1.5μM. Regarding the developmental delay, in 2012 Wang et al. showed a 24hr. exposure at the L2 stage was sufficient to induce DAF-16 translocation to the nucleus at doses as low as 1.0nM and as high as 0.2μM (Wang et al., 2012). DAF-16 is a transcription factor normally activated under conditions of environmental stress that functions to increase transcription of genes involved, stress resistance, dauer formation, and longevity at the cost of those for in reproductive development (Jensen, Galllo, and Riddle, 2006). The induction of this transcription factor at lower doses and a later stage than tested in this work provides one potential mechanistic explanation for our observed developmental delay. Analysis of DAF-16 translocation using our same model with the GR1352 strain may be an effective means to further test this hypothesis (Lee, Hench, and Ruvkun; 2001). Regarding the reproductive effects of TBT, prior work reveals the causal pathways may be similar to those of BPA. Cheng et al. showed that TBT exposure also shifted the double strand break repair process to the later pachytene as indicated by the number of RAD-51 positive nuclei in that stage of the gonadal arm. Furthermore, doses as low as 0.03μM TBT were found to significantly reduce the mean number of parental eggs laid and increased embryonic lethality (Cheng et al., 2014). While their results resemble ours, differences may be attributable to the exposure window. Cheng et al. began their 4-day reproductive toxicity study using nematodes that were age-synchronized at the L4-stage. In our model however, we began our
studies with age-synchronized L1- stage nematodes in order to chronically expose the developing germline, after which we examined reproductive changes on untreated media. The fact that our results demonstrated similar effects even in the absence of a direct exposure further highlights the lasting toxicity developmental TBT-exposure has on the growth and performance of the adult reproductive system and resultant progeny in *C. elegans*.

Fenthion significantly and dose-dependently reduced the number of eggs-laid and the number of hatches at all doses at and above 5.0μM. Nonlinear regression modeling of this data revealed an IC50\textsubscript{Egg-laying}= 3.7641μM (95% CI 1.563, 9.066; R²=0.1996). The wider confidence interval and poor R² value make this statistic less reliable, however this again may be due to the observed variability within and across treatment groups. The reduced egg-laying capacity may be due to lasting acetylcholinergic inhibition activity of fenthion as an organophosphate; since agonists such as nicotine are known to induce stimulatory effects on the involved muscle groups needed for this pathway (Waggoner et al., 2000). Additionally, fenthion did produce a shift in the day of maximal egg-laying for the 10.0μM, 50.0μM, and 100.0μM treated parent *C. elegans*, highlighting a potential developmental delay as seen in the other two studied biocides. Our data corroborate prior work showing alterations in egg-laying, viability, and development resulting from exposure to other organophosphate pesticides such as chlorpyrifos and monocrotophos (Ruan et al., 2011; Salim and Rajini, 2014; Roh and Choi, 2008). In mammals however, a maternally administered dose as high as approximately 2mM was needed to provoke spontaneous abortion and reduce litter size (Amara et al., 2014). Fenthion is still used in select states, including Florida. Our data
highlight that further research is still needed to determine the extent to which low doses affect the reproductive system and developing embryos, especially from the likely window of a chronic exposure.

Taken together, these data highlight that biocide exposures can affect the development of the reproductive system in \textit{C. elegans} and have lasting effects on fertility and fecundity, even after cessation of the exposure. Additionally, there currently exists ample room for further exploration of the mechanisms underlying this reprotoxicity in \textit{C. elegans}. Such experiments may be useful in establishing platforms to screen new biocides for potential reprotoxicity prior to their market dissemination.

\textbf{2.5.3 Specific Reprotoxic Effects of Cigarette Smoke and Its Constituents}

Interestingly, BAP had significant reprotoxic effects in \textit{C. elegans}, while CSE and nicotine did not. CSE induced no significant effect on egg-laying, however there were increased hatches and corresponding embryonic viability from the 50.0μM and 100.0μM exposure groups. Nicotine induced no significant changes in egg-laying, hatching, or embryonic viability across all doses. One reason why we may not have noticed any significant reprotoxic effects may be due to the cessation of exposure. Indeed, Zenzes noted reprotoxic effects in studies from actively smoking women (Zenzes et al., 1995; Zenzes et al., 1998). Furthermore, Green et al. demonstrated in 2009 that the levels of cotinine, a primary metabolite of nicotine, fell below the limits of detection in \textit{C. elegans} within 24 hours following exposure to cigarette smoke (Green et al., 2009). Relatedly, the results presented here appear to corroborate prior work concerning nicotine adaptation and recovery in \textit{C. elegans} by Waggoner et al. in 2000. There, acute nicotine exposure for 1 hour during adulthood was shown to increase egg-laying.
However, chronic nicotine exposure overnight conversely reduced egg-laying. These differences in acute and chronic effects were demonstrated to work via stimulation and adaptation, respectively, of the cholinergic-controlled egg-laying muscles in *C. elegans*. In one experiment in particular, egg-laying numbers from *C. elegans* exposed overnight recovered to to those of naïve, unexposed animals between 24-36 hours post-exposure (Waggoner et al., 2000). It is possible that our nematodes may have also recovered within the 24 hours post-removal from the chronic nicotine exposure. Additionally, our results also appear to resemble those of Smith Jr. et al. in 2013. Here, nicotine was administered at four doses ranging from 6.17μM to 194.5μM to L3-staged worms for 24 hours, after which they were allowed to recover and lay eggs. No significant differences were noted on the first 24 hours post exposure, but slight differences were noted 48 hours post exposure (Smith et al., 2013). Future analyses may be directed at maintaining a constant exposure model in *C. elegans* at higher doses than those tested here. Gonadal analysis of germ cell corpse number and RAD-51 positive foci as in Allard and Colaiácovo 2010 may provide more translatable mechanistic insight on CSE and nicotine’s potential reproductive toxicity in *C. elegans* by revealing if these agents may similarly act to reduce the germ cell pool as seen by Sobinoff et al, in 2013.

BAP dose-dependently and significantly reduced the number of eggs laid and the number of corresponding hatches at doses equal to and above 5.0μM. Nonlinear regression modeling of this data revealed an IC50_{Egg-laying} = 1.691μM (95% CI 0.9672, 2.958; R^2=0.3798). Our IC50_{Egg-laying} is slightly higher than previously reported for BAP in *C. elegans*, however this may be due to differences in exposure window. Sese, Grant, and Reid reported in 2009 an IC50_{Egg-laying} equal to approximately 0.233μM,
however this was in response to a 72-hour direct exposure (Sese, Grant, and Reid, 2009). We also found that parent *C. elegans* in the 5.0μM, 10.0μM, and 100.0μM exposure groups, BAP shifted the day of maximal egg-laying to the second day post-exposure. While it is possible this is indicative of a developmental delay, it is also possible this shift results from the lasting effects of BAP on the germ cells of the *C. elegans* gonad. BAP is known to induce DNA-adduct formation, which may contribute to a reduced number of viable oocytes available for fertilization during the first day post-exposure. Because BAP was one of the few compounds in this study to also significantly reduce embryonic viability, the later is a likely mechanistic explanation. The transparent nature of *C. elegans* would be particularly amenable to immunocytochemistry staining with #5D11 to detect PAH induced dioepoxide-DNA adducts (Zenzes et al., 1998). An increase in adducts would be expected to correlate well with an increase in the number of germ cell corpses and reduction in the number of mean eggs laid.

Taken together, these results indicate that CSE and nicotine exposure may need to be direct and constant in order to mediate its reprotoxic effects. BAP however is capable of having lasting effects in the absence of exposure. Because BAP is a constituent of cigarette smoke, and similar changes were not noted in CSE treated *C. elegans*, this may indicate that the exact concentration of BAP in CSE was too low to exert noticeable effects. To see if this is the case, CSE may be tested at higher concentrations and immunocytochemistry techniques mentioned previously may be applied to examine if BAP-mediated adduct formation is occurring. Because to date
only one other study details the effects of cigarette smoke on *C. elegans* (Green, 2009), this frontier is still relatively unexplored warranting further research.

### 2.5.4 Specific Reprotoxic Effects of Heavy Metals and Metalloids

Cadmium and arsenic(III)oxide both had significant effects on egg-laying, hatching, and embryonic percent viability. Chronic developmental exposure to 5.0μM and 10.0μM cadmium shifted the day of maximal egg-laying. Exposure to doses of 50.0μM and 100.0μM prevented worms from laying eggs at any point in the study, these P0 also had all died before the fourth day post-exposure. taken together these data suggest that developmental exposure to cadmium is capable of inducing a developmental delay or even complete sterility at high enough doses. Nonlinear regression modeling of egg-laying data revealed an $IC_{50_{Egg-laying}} = 2.085\mu M$ (95% CI 1.451, 2.998; $R^2=0.4825$). Our IC50 is noticeably lower than that reported in other studies, but again, this may be due to choice of exposure model. The prior reported EC50’s for similar endpoints evaluated in *C. elegans* are 176-192μM and 151μM (Boyd et al., 2010; Anderson, Boyd, and Williams, 2001). However, the study by Boyd examined a chronic exposure window beginning with L4-staged nematodes, where nematodes have already developed the majority of their reproductive organ system. Our study examines a chronic exposure window beginning at the L1-stage in which the earliest, 4 primordial germ cells that will divide and eventually become the gonad, are exposed. Taken together, our data highlight the heightened sensitivity of the developing reproductive organ system in *C. elegans* to the toxic effects of cadmium. Additionally, we demonstrate that the effects of increased infertility and reduced embryonic viability may persist well beyond cessation of the actual exposure.
Further evaluations of the relationship between cadmium and alterations in development and reproductive capacity may be best directed at better understanding the relationship between metallothioneins and the daf-2 insulin-like signaling pathway in *C. elegans*. DAF-2 is the *C. elegans* insulin receptor responsible for inhibition of DAF-16 stress resistance activities under normal conditions. Two types of metallothioneins, MT1 and MT2, are involved in detoxification processes that take place after metal exposure. Prior work by Barsyte et al. in 2001 revealed that on a daf-2 mutant background the LC50 in response to cadmium treatment was shifted higher and MT expression was doubled; indicating increased resistance compared to wild-type worms. These data highlighted the role of DAF-16 in mediating metal-induced toxicity (Barsyte, Lovejoy, and Lithgow, 2001). Because DAF-16 stress resistance activity operates while compromising reproductive development, an analysis using the the GR1352 strain may help to temporally visualize changed in DAF-16 localization in response to chronic developmental exposure (Lee, Hench, and Ruvkun, 2001). Under our same model, continued examination of DAF-16 translocation to the nucleus in P0 nematodes throughout the four days post-exposure would confirm one potential mechanistic pathways for our noted, lasting effects on reproduction.

Chronic exposure to arsenic(III)oxide significantly increased egg-laying in the 0.1μM exposure group and decreased egg-laying in the 50.0μM and 100.0μM exposure groups. Nonlinear regression modeling of egg-laying data revealed an IC50_{Egg-laying} = 16.07μM (95% CI 7.44, 34.70; R^2=0.196). The wide confidence interval and low R^2 value however, make this statistic difficult to rely on. Reductions in hatched larvae were only noted from the 50.0μM and 100.0μM exposure groups. Embryonic viability was
similarly increased in the 0.1µM exposure group and decreased egg-laying in the 50.0µM and 100.0µM exposure groups. Nonlinear regression modeling of embryonic viability data revealed an LD50 = 13.41µM (95% CI 8.694, 20.69; R²=0.4245). This was the only compound for which we could successfully calculate an embryonic LD50. The reduction in both eggs laid and embryonic viability suggest that higher doses of arsenic(III)oxide target the developing embryo and perhaps the germ cell pool as well. Evidence exists that the negative effects of arsenic(III)oxide on reproduction may mechanistically be similar to those of cadmium. In 2013 Schmeisser et al. found that low-dose exposure to 0.1µM sodium arsenite increased lifespan in C. elegans via a DAF-16 mediated pathway (Schmeisser et al., 2013). Because DAF-16 is similarly implicated in arsenic-mediated toxicity, future work using our same exposure model may be directed at increasing the doses of arsenic(III)oxide tested in order to see if the reproductive and developmental delays noted in response to cadmium exposure can be achieved.

Taken together, the results of this study indicate the noticeable differences in C. elegans sensitivities to distinct metal species. The significant reduction in embryonic percent viability highlights the embryo as a target for disruption. Further analysis into the role of DAF-16, MT1, and MT2, would provide enhanced mechanistic insight into the lasting reprotoxic consequences of these chronic developmental exposures.

2.5.5 Reprotoxicity Conclusions

We have established a C. elegans model system with an exposure window encompassing exclusively the period of reproductive organ development in the nematode. Because of this design, we are uniquely situated to address the toxic effects
of chronic larval exposure to environmental teratogens. The results of this study saliently highlight the potential of certain teratogens to have lasting effects on development, reproduction, and embryonic viability even after cessation of the exposure. Future work may best be directed at analyzing related molecular signaling pathways on a more individualized-compound basis. It may be interesting to examine the effects of BAP for example, using immunocytochemistry techniques from clinical studies by Zenzes et al., 1995 alongside gonadal cell progression analyses as performed by Allard and Colaiácovo 2010. For cadmium and arsenic(III)oxide however, it may be more interesting a route to investigate the role of DAF-16 in a manner used to evaluate the metal-containing biocide TBT by Wang et al., 2012. Further mechanistic work is needed to shed light on the magnitude of physiological changes resulting from teratogen exposure and highlight targets for therapeutic intervention.

Importantly, we plan to use the results of this study for selection of the proper doses for our obesogen screen outlined in Chapter 3. In order to appropriately model the Fetal Origins of Health and Disease Hypothesis, doses below the threshold for significant embryotoxic effects should be chosen. This is because the effective dose to induce offspring disease in later life should in theory, be the one that did not cause its death in utero. We have chosen the following doses for our selected teratogens to assess in future work based on the results of this current study: 10μM BPA, 10μM DES, 0.1μM TBT-Cl, 0.1μM fenthion, 0.1μM triclosan, 1.0μM CSE, 0.5μM nicotine, 0.5μM benzo-α-pyrene, 0.5μM Cd-Cl, and 0.5μM As(III)O. These doses allowed for the production of a sufficient number of viable offspring to be analyzed in the obesogen screen.
2.6 References


Figure 2.1: An illustrated schematic of the performed workflow assessing reproductive toxicity. where (1) the age-synchronized parental *C. elegans* are plated onto NGM containing exposure or controls for 48 hours after which (2) they are moved to row A of a 24-well plate containing untreated NGM where (3) each day they are moved to the row below for 4 days.
Figure 2.2: Bisphenol-A induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post BPA-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure.
Figure 2.3: Bisphenol-A affects egg-laying and larval hatching. Box plots of egg-laying (A) and hatched larvae (B) data from P0 C. elegans on the first day post-BPA exposure reveal medians and quartile spread. (A) 0.5μM BPA exposed P0 lay significantly fewer mean eggs, while 50.0μM BPA exposed P0 laid significantly more eggs (*p<0.05). (B) 100.0μM BPA exposed P0 had the highest significant overall mean number of hatched larvae (*p<0.05). Circles indicate outliers.
Figure 2.4: Bisphenol-A does not affect embryonic viability. Box plot of embryonic percent viability data from the first day post-BPA exposure reveals medians and quartile spread. No significant differences were noted in any of the groups when compared to vehicle control. Circles indicate outliers.
Figure 2.5: Diethylstilbestrol induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post DES-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure.
**Figure 2.6: Diethylstilbestrol affects egg-laying.** Box plots of egg-laying (A) and hatched larvae (B) data from P0 *C. elegans* on the first day post-DES exposure reveal medians and quartile spread. (A) 50.0μM and 100.0μM DES exposed P0 lay significantly fewer mean eggs (*p<0.05*). (B) 100.0μM DES exposed P0 had the highest significant overall mean number of hatched larvae (*p<0.05*). Circles indicate outliers.
Figure 2.7: Diethylstilbestrol does not affect embryonic viability. Box plot of embryonic percent viability data from the first day post-DES exposure reveals medians and quartile spread. No significant differences were noted in any of the groups when compared to vehicle control. Circles indicate outliers.
Figure 2.8: Triclosan induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post triclosan-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure for P0 exposed to 0.1-1.0μM. P0 exposed to 5.0μM and 10.0μM triclosan laid more eggs on day two. P0 exposed to 50.0uM and 100.0μM laid no eggs.
Figure 2.9: Triclosan affects egg-laying and hatching. Box plots of egg-laying (A) and hatched larvae (B) data from P0 C. elegans on the first day post-triclosan exposure reveal medians and quartile spread. (A) 5.0μM and 10.0μM triclosan exposed P0 laid significantly fewer mean eggs, while 50.0μM and 100.0μM triclosan exposed P0 laid no eggs and consequently had no hatches. (B) 5.0μM triclosan exposed P0 had the lowest significant overall mean number of hatched larvae (†p<0.05). Circles and asterisks indicate outliers 1 and 2 SE beyond the mean.
Figure 2.10: Triclosan does not affect embryonic viability. Box plot of embryonic percent viability data from the first day post-triclosan exposure reveals medians and quartile spread. No significant differences were noted in any groups when compared to vehicle control. Circles indicate outliers.

Figure 2.11: Triclosan reduces egg-laying. A nonlinear regression of normalized egg-laying data plotted against log[μM Triclosan] shows a 50% reduction in egg-laying IC50=4.262μM (95% CI [2.962, 6.748], R²=0.4727).
Figure 2.12: Tributyltin induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post TBT-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the second day post-exposure for P0 exposed to 0.5μM TBT and above.
Figure 2.13: Tributyltin affects egg-laying and hatching. Box plots of egg-laying (A) and hatched larvae (B) data from P0 C. elegans on the first day post-TBT exposure reveal medians and quartile spread. (A) P0 exposed to doses of 0.5μM and 1.0μM TBT laid significantly fewer mean eggs. P0 exposed to 5.0μM and 10.0μM TBT laid no eggs and consequently had no hatches. (B) P0 exposed to doses of 0.5μM and 1.0μM TBT or greater also had significantly fewer mean hatched larvae. (*p<0.05). Circles indicate outliers.
**Figure 2.14: Tributyltin affects embryonic viability.** Box plot of embryonic percent viability data from the first day post-TBT exposure reveals medians and quartile spread. Embryos of P0 exposed to doses of 0.5μM TBT had significantly reduced viability compared to those of vehicle control (*p<0.05). P0 exposed to 5.0μM and 10.0μM TBT laid no eggs and thus were not subject to comparison.

**Figure 2.15: Tributyltin reduced egg-laying.** A nonlinear regression of normalized egg-laying data plotted against log[μM TBT] shows a 50% reduction in egg-laying IC50=0.6402μM (95% CI [0.5326, 0.7697], R²=0.7311).
Figure 2.16: Fenthion induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post fenthion-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure for P0 exposed to 0.1-5.0μM. P0 exposed to 10.0μM-100.0μM fenthion laid more eggs on day two.
Figure 2.17: Fenthion affects egg-laying and hatching. Box plots of egg-laying (A) and hatched larvae (B) data from P0 *C. elegans* on the first day post-fenthion exposure reveal medians and quartile spread. (A) P0 exposed to doses of 5.0μM of fenthion and above laid significantly fewer mean eggs. (B) These same P0 also had significantly fewer mean hatched larvae. (*p<0.05*). Circles indicate outliers.
**Figure 2.18: Fenthion does not affect embryonic viability.** Box plot of embryonic percent viability data from the first day post-fenthion exposure reveals medians and quartile spread. No significant differences were noted in any of the groups when compared to vehicle control.

**Figure 2.19: Fenthion reduces egg-laying.** A nonlinear regression of normalized egg-laying data plotted against log [μM Fenthion] shows a 50% reduction in egg-laying IC50=3.764 μM (95% CI [1.563, 9.066], R²=0.1996).
Figure 2.20: Cigarette smoke extract induces egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post CSE-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure.
Figure 2.21: Cigarette smoke extract affects hatching but not egg-laying. Box plots of egg-laying (A) and hatched larvae (B) data from P0 *C. elegans* on the first day post-CSE exposure reveal medians and quartile spread. (A) No significant differences were noted in mean egg-laying across CSE treated P0 when compared to vehicle control (B) P0 exposed to doses of 50.0μM and 100.0μM CSE however had significantly greater mean hatched larvae when compared to vehicle control (*p*<0.05). Circles indicate outliers.
Figure 2.22: Cigarette smoke extract affects embryonic viability. Box plot of embryonic percent viability data from the first day post-CSE exposure reveals medians and quartile spread. Embryos of P0 exposed to doses of 50.0μM and 100.0μM CSE had significantly greater mean embryonic viability than those of vehicle controls (*p<0.05).
Figure 2.23: Nicotine induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post nicotine-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure.
Figure 2.24: Nicotine does not affect egg-laying or hatching. Box plots of egg-laying (A) and hatched larvae (B) data from P0 C. elegans on the first day post-nicotine exposure reveal medians and quartile spread. (A) No significant differences were seen in mean egg-laying across nicotine treated P0 compared to vehicle control (B) No significant differences were seen in mean hatched larvae across nicotine treated P0 compared to vehicle control. Circles indicate outliers.
Figure 2.25: Nicotine does not affect embryonic viability. Box plot of embryonic percent viability data from the first day post-nicotine exposure reveals medians and quartile spread. No significant differences were noted in any of the groups when compared to vehicle control. Circles indicate outliers 1 SE beyond the mean.
Figure 2.26: Benzo-α-pyrene induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post BAP-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure for P0 exposed to 0.1-1.0μM. P0 exposed to 5.0μM, 10.0μM, and 100.0μM laid maximal eggs on day two. P0 exposed to 100.0μM had maximal hatches on day two.
Figure 2.27: Benzo-α-pyrene affects egg-laying and hatching. Box plots of egg-laying (A) and hatched larvae (B) data from P0 C. elegans on the first day post-BAP exposure reveal medians and quartile spread. (A) P0 exposed to doses of 5.0μM BAP and above laid significantly fewer mean eggs than vehicle control (B) P0 exposed to doses of 5.0μM, 10.0μM, and 100.0μM BAP had significantly fewer mean hatched larvae when compared to vehicle control. (*p<0.05). Circles indicate outliers.
**Figure 2.28: Benzo-α-pyrene does not affect embryonic viability.** Box plot of embryonic percent viability data from the first day post-BAP exposure reveals medians and quartile spread. No significant differences were noted in any of the groups when compared to vehicle control. Circles indicate outliers 1 SE beyond the mean.

**Figure 2.29: Benzo-α-pyrene reduces egg-laying.** A nonlinear regression of normalized egg-laying data plotted against log[μM BAP] shows a 50% reduction in egg-laying IC50=1.691 μM (95% CI [0.9672, 2.958], R²=0.3798).
Figure 2.30: Cadmium induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post cadmium-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure for P0 exposed to 0.1-1.0μM. P0 exposed to 5.0μM and 10.0μM cadmium laid more eggs on day two. P0 exposed to 50.0μM and 100.0μM laid no eggs.
Figure 2.31: Cadmium affects egg-laying and hatching. Box plots of egg-laying (A) and hatched larvae (B) data from P0 C. elegans on the first day post-cadmium exposure reveal medians and quartile spread. (A) P0 exposed to doses of 5.0μM and 10.0μM cadmium laid significantly fewer mean eggs than vehicle control. P0 exposed to 50.0μM and 100.0μM cadmium laid no eggs and consequently had no corresponding hatches. (B) P0 exposed to 5.0μM and 10.0μM had significantly fewer mean hatched larvae when compared to vehicle control. (†p<0.05). Circles and asterisks indicate outliers 1 and 2 SE beyond the mean.
Figure 2.32: Cadmium affects embryonic viability. Box plot of embryonic percent viability data from the first day post-cadmium exposure reveals medians and quartile spread. Embryos of P0 exposed to 0.1μM, 1.0μM, 5.0μM and 10.0μM cadmium had significantly reduced embryonic viability compared to those from vehicle control. P0 exposed to 50.0μM and 100.0μM cadmium laid no eggs and were thus not subject to comparison. (*p<0.05). Circles indicate outliers 1 SE beyond the mean.

Figure 2.33: Cadmium reduces egg-laying. A nonlinear regression of normalized egg-laying data plotted against log[μM Cd] shows a 50% reduction in egg-laying IC50=2.086 μM (95% CI [1.451, 2.998], R²=0.4825).
Figure 2.34: Arsenic(III)oxide induced egg-laying and larval hatching trends over 4 days. The mean number of eggs laid (A) and hatched larvae (B) from P0 C. elegans over the first four days post As(III)O-exposure, error bars represent SEM. Maximal egg-laying and hatched larvae were noted on the first day post-exposure.
Figure 2.35: Arsenic(III)oxide affects egg-laying and hatching. Box plots of egg-laying (A) and hatched larvae (B) data from P0 C. elegans on the first day post-As(III)O exposure reveal medians and quartile spread. (A) P0 exposed to doses of 0.1μM As(III)O laid significantly greater mean eggs than vehicle control. P0 exposed to 50.0μM and 100.0μM As(III)O laid significantly fewer mean eggs than vehicle control. (B) P0 exposed to 50.0μM and 100.0μM had significantly fewer mean hatched larvae when compared to vehicle control. (‡p<0.05). Circles and asterisks indicate outliers 1 and 2 SE beyond the mean.
Figure 2.36: Arsenic(III) oxide affects embryonic viability. Box plot of embryonic percent viability data from the first day post-As(III)O exposure reveals medians and quartile spread. Embryos of P0 exposed to 0.5μM As(III)O had significantly increased embryonic viability compared to those from vehicle controls. Embryos of P0 exposed to 50.0μM As(III)O had significantly reduced embryonic viability compared to those from vehicle control. (†p<0.05). Circles and asterisks indicate outliers 1 and 2 SE beyond the mean.
Figure 2.37: Arsenic(III)oxide reduces egg-laying and increases embryonic lethality. (A) A nonlinear regression of normalized egg-laying data plotted against log[μM As(III)O] shows a 50% reduction in egg-laying IC50=16.07 μM (95% CI [7.440, 34.7], R²=0.1960). (B) A nonlinear regression of normalized embryonic lethality data plotted against log[μM As(III)O] shows an LD50=13.41 μM (95% CI [8.694, 20.69], R²=0.4245).
Table 2.1: Summary table for significant increases or decreases induced by P0 exposure to the listed compounds. Colors are indicative of grouping such that blue = estrogen mimics, orange = biocides, green = cigarette smoke and relevant constituents, and yellow = heavy metals and metalloids.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Day of Maximal Egg-laying</th>
<th>Average Eggs Laid Day 1</th>
<th>Average Hatches from Day 1 Eggs</th>
<th>Average Embryonic Viability on Day 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>BPA</td>
<td>X↑</td>
<td>↓X↑</td>
<td>X↑</td>
<td></td>
</tr>
<tr>
<td>DES</td>
<td></td>
<td>↓X</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Triclosan</td>
<td>X↑</td>
<td>↓X</td>
<td>↓X</td>
<td></td>
</tr>
<tr>
<td>TBT</td>
<td>X↑</td>
<td>↓X</td>
<td>↓X</td>
<td>↓X</td>
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<tr>
<td>Fenthion</td>
<td>X↑</td>
<td>↓X</td>
<td>↓X</td>
<td></td>
</tr>
<tr>
<td>CSE</td>
<td></td>
<td>X↑</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotine</td>
<td></td>
<td></td>
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<tr>
<td>BAP</td>
<td>X↑</td>
<td>↓X</td>
<td>↓X</td>
<td></td>
</tr>
<tr>
<td>Cadmium</td>
<td>X↑</td>
<td>↓X</td>
<td>↓X</td>
<td>↓X</td>
</tr>
<tr>
<td>Arsenic(III)oxide</td>
<td></td>
<td>↓X↑</td>
<td>↓X</td>
<td>↓X</td>
</tr>
</tbody>
</table>
CHAPTER THREE
METABOLIC EFFECTS OF SELECT DEVELOPMENTAL TERATOGENS

3.1 Prevalence and Impact of Obesity

According to NIH-curated statistics, over the past 3 decades the prevalence of adult obesity in the United States has risen more than two-fold. Furthermore, a startling 31.8% of all children ages 2-19 years are considered overweight or obese. This disease imparts a host of physical, psychological, and economic challenges for those affected. Obesity is associated with increased susceptibility to an assortment of other diseases including type 2 diabetes, cancer, and stroke (NIH, 2012). Due to these risks, in adults less than 70 years old obesity is the third-leading lifestyle based cause of death after tobacco smoke and high blood pressure (Danaei et al., 2009). Obese individuals exhibit a higher prevalence of depressive behavior compared to the general population and are more likely to report reduced quality of life as a result of their excessive weight (Jagielski et al., 2014). The individual costs of obesity also add up. On a yearly basis an overweight person will spend approximately $400-500 and an obese person $2500-5000 as a result of disease-related death, lost wages, and health insurance (Dor et al., 2010). Furthermore, the social burden of obesity related medical care by 2018 is expected to be $344 billion (UHF and APHAPP, 2009).

Mentioned earlier, the increased interest in the causes of obesity led the National Institute of Environmental Health Sciences to initiate an interdisciplinary workshop in an effort to review the literature and highlight potential developmental programmers for this
disease (Thayer, Heindel, & Bucher, 2012). The goal of this study is to address putative obesogens that are common in our environment, specifically within the context of the Fetal Origins of Health and Disease Hypothesis. In particular, we aim to demonstrate that obesogens exert their effects of developmental programming during a very early window of embryonic development in the model organism *Caenorhabditis elegans*.

3.2 Hypothesis

*C. elegans* can be used to screen sub-embryonic lethal doses of putative obesogens during early embryonic development; these will increase the body area Oil Red-O stained for lipids once they have reached adulthood. The model may also be used to detect changes in energy balance via pharyngeal pumping or body thrashing rates.

3.3 Methods

3.3.1 *C. elegans* Strain and Colony Maintenance

*C. elegans* were maintained as described in 2.3.1. Similar to section 2.3.1, the *E. coli* strain of OP50 was used as the feeding strain as it is a uracil auxotroph. This characteristic allowed the bacteria to grow more slowly, preventing the quick formation of a thick lawn of bacteria that may occlude the visualization of embryos and hatched larvae.

3.3.2 Developmental-synchronization

*C. elegans* age-synchronization was performed as described in 2.3.2.

3.3.3 Chemicals

All chemicals used were acquired as described in 2.3.3. 400μL of each teratogen DMSO stock was added directly to 39.6mL of NGM lite agar and was mixed by vortexer on a low setting in order minimize generating bubbles. This was done to
generate the following dilutions: 10μM BPA, 10μM DES, 0.1μM TBT-Cl, 0.1μM Fenthion, 0.1μM Triclosan, 1.0μM CSE, 0.5μM Nicotine, 0.5μM Benzo-a-pyrene, 0.5μM Cd-Cl, and 0.5μM As(III)O, in 0.01% DMSO in NGM agar. 400μL of 100% DMSO was added to 39.6mL of NGM to generate 0.01% DMSO vehicle control (0 μM) plates. Approximately 6mL of each dilution in NGM was poured into each 60mm petri dish for each exposure group.

As described earlier, because these teratogens pose a threat to human health and the environment, the necessary precautions were taken under the recommendation of the University of South Florida’s Environmental Health and Safety Department to minimize and properly dispose of all waste produced in the generation of these dilutions.

3.3.4 Exposure Design

On the first day of the experiment, approximately 400 adult nematodes were rinsed from six, mixed developmental stage nematode plates (60mm). These worms were developmentally-synchronized with alkaline bleach and left to hatch as described above. On this day the experimental NGM plates were seeded with 50μL of OP50 feeding stock, let to dry in a closed hood, and incubated for approximately 16-18 hours overnight at 25°C. All control and experimental NGM plates were coded. All experiments were performed and data were collected double blind with the observer unaware to experimental conditions. Data was decoded after the experiment was performed once it was collected and submitted to the coder.

On the second day, the vial of L1 larvae was incubated at 4°C for 5 minutes, centrifuged for 2 minutes at 1000 RPM, and excess M9 buffer was aspirated to
approximately the 0.5mL mark. The L1 pellet was resuspended by gently tapping the bottom of the vial or vortexer on a low setting. Three 10µL drops of the resuspended worm sample were pipetted onto the lid of an empty petri dish. The number of L1 nematodes were counted in each drop and averaged to estimate the number of L1 larvae per microliter in the sample suspension. 10-50µL M9 buffer containing at least 50 L1 larvae in total was placed onto each plate and allowed to dry under a closed hood. Dry plates were sealed with parafilm and placed into the low-temperature incubator for 57 hours at 25°C.

On the third day of the experiment, untreated NGM plates were seeded with 1X OP50 feeding stock spread over the whole surface of the plate and put to incubate for 16-18 hours at 25°C. The volumes of OP50 feeding stock used were 50µL for a 60mm plate or 100µL for a 100mm plate.

On day four of the experiment at the 57-hour mark, P0 C. elegans were moved from the exposure NGM plate to an untreated plate and left to lay eggs in a dark incubator for 3 hours at 25°C. For a 60mm plate, 20-40 P0 nematodes were moved to an egg-laying plate. For a 100mm plate, 70-100 P0 were moved to an egg-laying plate. After 5 hours the P0 C. elegans were individually picked and removed from the plate. The remaining embryos remained on the plate to hatch and grow for 60 hours at at 25°C, after which these offspring (F1) were metabolically phenotyped.

3.3.5 Oil Red-O Lipid Staining

Approximately 200-300 adult nematode F1 were collected into a 1.5mL centrifuge tube and washed 3 times with cold 1X PBS. Incubation for roughly a minute on ice or a 3-5 second centrifugation on a Spectrafuge mini centrifuge was used to
settle the worms to the bottom of the tube between washes (C1301, Labnet International Inc., Edison, NJ). After the final wash, the remaining PBS was aspirated to the 0.1mL mark on the tube. To permeabilize the cuticle, the following was added to remaining sample: 20µl 1X PBS, 24µL of 10% paraformaldehyde, and 96µL 2X Modified Ruvkun’s Worm Buffer (MRWB) (160mM KCl, 40mM NaCl, 14mM Na$_2$EGTA, 1mM spermidine, 0.4mM spermine, 10mM PIPES (pH 7.4), and 0.2% beta-mercaptoethanol; sterilized via 0.22µM filter). Samples were rocked gently on a three-dimensional shaking motion rotator for 2 hours (GyroTwister GX-1000, LabNet International, Edison, NJ, USA; or Adams Nutator, Becton Dickson and Company, Franklin Lakes, NJ). While the nematode samples were being fixed, the Oil Red-O stain was prepared. Stock Oil Red-O (0.5mg in 100mL 100% isopropanol) was adjusted to 60% with ultrapure water in a 15mL or 50mL conical vial at an appropriate volume required to stain all samples (12989, Alfa Aesar, Ward Hill, MA). This diluted stain was equilibrated on the same shaking motion rotator for at least one hour. After the samples were permealized for two hours, they were taken off the shaker and the nematodes were allowed to settle to the bottom of the tube. These samples were washed 3 additional times with room temperature 1X PBS before dehydration. Three dehydration steps were taken at 10%, 30%, and 60% isopropanol in 1X PBS for 15 minutes. After the final dehydration, the 60% Oil Red-O dye was added. Freshly diluted and filtered Oil Red-O dye yielded optimal results. The best filter to use for freshly diluted and equilibrated Oil Red-O dye was a 0.22µm cellulose nitrate filter. 0.22µm cellulose acetate and mixed cellulose ester filters resulted in stained worms that showed a large amount of dye precipitate and were therefore not analyzable. Once the 60% Oil Red-O dye was added, the samples
were placed back onto the shaking motion rotator for approximately 22-24 hours overnight. The next day, the tubes were removed from the shaking motion rotator and the nematodes were allowed to settle to the bottom of the tube. After settling, the samples underwent 3 rehydration steps at 60%, 30% and 10% isopropanol in 1X PBS for 15 minutes. After the final wash and aspiration, 25μL of 1X PBS with 0.01% Triton-X was added to the nematode sample. The inclusion of Triton-X is essential, as without it the majority of the nematode sample will adhere to the inside of the plastic pipette tip upon transfer to an agarose slide pad. 3% agarose slide pads were made by placing three strips of laboratory tape to the top and bottom of a glass slide, pipetting molten 3% agarose solution in the center of the slide between the tape strips, and placing a clean, clear glass slide on top to flatten the pad until the it was solidified. All slides and coverslips were cleaned with 100% or 95% ethanol and dried using low lint tissue prior to use. Once the agarose slide pads had solidified, 25-50μL of the nematode sample was pipetted onto the pad and covered with a glass coverslip. Excess liquid was wicked off with a low lint tissue and excess agarose was trimmed with a razor blade. Clear, non-fluorescing nail-polish was used to seal the slide pad; at least three coats were required to completely seal the slide. The slides were imaged within 3 days, and kept in a humidified box at 25°C during any interim. Slides were imaged on a Nikon Eclipse E100 at 20X using a QImaging Retiga 1300 camera with RGB slider color filter (QImaging, Surrey, BC).

3.3.6 Image Analysis

Nematode images taken in segments were seamed in Adobe Illustrator and analyzed in Image Pro (Media Cybernetics Inc., Rockville, MD). First, the green
channel was isolated as the red dye absorbs in the green wavelength of light (Yen, 2010). Once the green channel was isolated, the nematode or relevant body segment (head, proximal, distal, or tail region) was outlined as an area of interest (AOI). Since a 3-dimensional object image was being assessed, the background correction tool was used to automatically remove any potentially confounding background shadows in the image. The AOI in the background corrected image was highlighted and inverted in order to assess total staining area. An arbitrary range was established by examining several nematode images in a replicate. First, the background-corrected green channel was pulled out and the thresholds for pixel intensities were manually adjusted to produce a mask that by visual comparison, best matched the stained area in the color image. This resulted in a range of intensity values that would be counted as stained area within the outlined nematode. In our experiments presented here, the ranges used were either 100-255, or 125-255. The select arbitrary range was consistently used across all groups within a replicate to count the number of Oil Red-O stained pixels within the AOI. In the assessment of the staining across the whole body, the AOI was then converted to an object and the total pixels in the AOI were recorded, this reflected the area of the whole worm.

**3.3.7 Pharyngeal Pumping Assay**

The day prior to performing the assay, pharyngeal pumping assay plates were made by filling 35mm petri dishes with 2mL of NGM lite (N1005, USBiologicals, Salem, MA). These were seeded with 35μL of 1X OP50 and incubated at 25°C overnight. The next day, 15-20 nematodes were picked and moved to an appropriately labeled pharyngeal pumping plate and returned to the 25°C for at least one hour to adjust to the
After adjusting, movies were recorded with the CellSense® software on an Olympus SZX16 stereomicroscope. Two movies were taken for each individual worm to control for intra-worm variability. Movies were recorded in greyscale. After both movies had been taken for each worm, that worm was individually picked and moved to an unseeded 35mm NGM plate, these were later used in the body thrashing assay. Once all movies were recorded, these were cropped to the 10-second mark using Quicktime 7 Pro®. Quicktime 7 Pro® was also used to visualize movies as it allowed enhancement of video playback speed, light, and contrast which better aided pharynx visualization in the nematode. Change in grinder position was regarded as a pharyngeal pump (Avery and Shtonda 2003). Each movie was watched and the number of pharyngeal pumps recorded twice by a blind rater.

### 3.3.8 Body Thrashing Assay

The day prior to performing the assay, body thrashing assay plates were made by filling 35mm petri plates with 2mL NGM lite as in 3.3.9. These plates were left unseeded and incubated at 4°C overnight. On the day the assay was performed, these plates and 1-2mL of M9 buffer (see 2.3.2) were brought to room temperature prior to beginning the assay. Nematodes that were used in the pharyngeal pumping assay were gradually added to an appropriately labeled unseeded NGM plate to remove excess bacteria (Nawa et al., 2012). In some instances, nematodes placed on the unseeded plate escaped to the edges and died. Occasionally, nematodes were accidentally killed during the transfer processes. These two factors contributed to the reduced sample size in this assay compared to that of the pharyngeal pumping assay. A clear, unfrosted glass slide was cleaned with 70% ethanol and dried with low-lint
tissue. A 10μL drop of room temperature M9 buffer was placed on the slide, into which one individual nematode was picked from the unseeded plate and placed. Nematodes were allowed to adjust for a minute prior to movie recording. After adjusting, movies were recorded with the CellSense® software on the same Olympus SZX16 stereomicroscope. Only one movie was taken per nematode. Movies were recorded in greyscale. Once all movies were recorded, these were cropped to the 10-second mark using Quicktime 7 Pro® as in 3.3.8. A change in the body bending direction from midline was regarded as one thrash (Miller et al., 1996). Each movie was watched and the number of body thrashes recorded twice by a blind rater.

3.3.9 Statistical Analysis

Statistical Package for the Social Sciences (IBM SPSS Version 23) was used to perform descriptive and inferential statistical analyses, and generate corresponding figures. All data were analyzed via one-way ANOVAs followed by LSD post-hoc analysis or Spearman rho correlations. Plots of average percentages of body stained for each obesogen against its corresponding region-specific contribution to overall stained area were made to visualize the relationship.

3.4 Results

3.4.1 Developmental Obesogen Exposure Affects Stained Area and Percent of Area of Body Stained with Oil Red-O

Total sample size for each group across 5 experimental replicates ranged from 143-165 nematodes/group. A total number of 1703 nematodes was assessed in this experiment. Developmental exposure to obesogens at sub-embryonic lethal dosages induced no significant effects on total offspring body size when compared to 0.01%
DMSO vehicle control exposed offspring, whose total area was on average 219930 pixels\(^2\). Stained area was significantly increased in offspring developmentally exposed to BPA, DES, triclosan, TBT, fenthion, CSE, and As(III)O compared to 0.01% DMSO vehicle control offspring (ANOVA F(10,1692)=2.45. p<0.01) (Figure 3.2B). Exposed offspring stained areas were 1.15-1.28 times greater in size compared to vehicle offspring stained areas (10035-18866 pixels\(^2\) greater, Table 3.1). The stained areas for offspring exposed developmentally to BAP and nicotine were also greater than those for vehicle controls, however only at p-values that approached significance (p=0.075 and 0.053, respectively). Offspring exposed to all putative obesogens with the exception of cadmium displayed significantly increased percentages of body staining with Oil Red-O when compared to vehicle control-exposed offspring (ANOVA F(10,1692)=2.50. p<0.01) (Figure 3.3). Visible differences in storage deposition across the nematode body prompted us to further examine this phenomenon in greater detail.

### 3.4.2 Developmental Obesogen Exposure Affects the Percent Contribution of Body Regions to Overall Stained Area

The stained area was further assessed in obesogen exposed offspring according to 4 body regions: head, anterior gonad, posterior gonad, and tail regions (Figure 3.4). The percent contribution of each region to the overall area stained stained with Oil Red-O was then examined (Figure 3.5). Differences were best visualized when graphed specifically to region (Figures 3.6 and 3.7). Offspring developmentally exposed to obesogens exhibited visible differences across tail region when compared to vehicle control exposed offspring, these however, were not statistically significant. Offspring in the triclosan and fenthion exposed groups, showed significantly increased contribution
of the posterior gonad region to the overall stained area (ANOVA F(10,1692)=2.43, p<0.01). Interestingly, offspring exposed developmentally to BPA of triclosan did show significantly reduced contributions of the anterior gonad region to the overall stained area (ANOVA F(10,1692)=2.32, p<0.01) (Figure 3.7A). These same offspring developmentally exposed to BPA also exhibited a significantly increased contribution of the head region to the overall stained area (ANOVA F(10,1692)=2.49, p<0.01). Specifically, BPA exposed offspring exhibited an approximately 1.7X greater contribution of their head region to overall body staining than did vehicle exposed controls. While visibly similar effects were noted in the TBT and nicotine treated groups, these associations were again, not statistically significant (Figure 3.7B).

A potential relationship between stained area and depot expansion prompted the recommendation to analyze the two variable in relation to each other using Spearman's rho correlation (Figure 3.8). Initial qualitative observations revealed that most of the obesogen groups, exhibiting higher percentages of body area stained with Oil Red-O compared to vehicle, also had comparably greater contributions by their head or tail regions (rho=0.73 and 0.425, p<0.01 respectively). This was not true for CSE, however this group did have a comparably larger contribution to the overall stained area in the posterior gonad region. We noticed also that certain clustering events were taking place across the four plots. For instance, highlighted with the green ovals, CSE, nicotine, and BAP tended to cluster closer to each other according to their percent of body stained and at regional contribution levels comparable to those seen by the vehicle group in all plots. The purple ovals however show that BPA, DES, fenthion, and triclosan clustered close to each other. These had similar percentages of body area
stained with Oil Red-O, but they also had similarly increased regional contributions by their posterior gonad and tail regions to overall stained area. Furthermore, this cluster exhibited similarly decreased contributions from the anterior gonad region. Fenthion appeared to follow a regional distribution pattern similar to that exhibited by the estrogen mimics, but did not cluster close by due to its reduced average percent of body stained. TBT and As(III)O the two groups with the highest percentages of body stained, did not show any clear clustering or pattern.

3.4.3 Developmental Obesogen Exposure Reduces Offspring Pharyngeal Pumping Rate with No Effects on Body Thrashing Rate

For the pharyngeal pumping assay, the total nematodes examined per group across 5 experimental replicates ranged from 41-50 nematodes/group. A total number of 519 nematodes were assessed in this experiment. Developmental exposure to CSE and As(III)O induced significant reductions in offspring pharyngeal pumping rate when compared to vehicle exposed control offspring (ANOVA F(10,508)=4.05, p<0.001) (Fig 3.8). These exposed offspring had approximately 29-39 fewer pumps per minute than control offspring (Table 3.3). For the body thrashing assay, the total nematodes examined per group ranged from 41-50 nematodes/group. A total number of 502 nematodes were assessed in this experiment. Slightly fewer nematodes were present for the body thrashing assay than the pharyngeal pumping assay as some would escape from the unseeded plate during the performance of the pharyngeal pumping assay or were injured during transfer. Although exposed offspring in all groups showed lower body thrashing rates compared to vehicle control offspring (Table 3.4, Figure 3.9), these differences were not statistically significant.
3.5 Discussion

3.5.1 Obesogens Increase Overall Lipid Staining and Alter Depot Contribution in *C. elegans*

Developmental exposure to all of the putative obesogens except nicotine, BAP, and cadmium significantly increased the total Oil Red-O stained body area in the resultant offspring. An increase in the stained area of nicotine and BAP exposed groups approached statistical significance. Developmental exposure to all of the proposed obesogens except cadmium also significantly increased the percent of body stained in offspring. The observed changes in lipid staining across the nematode body are likely not due to a diverse microbiome, as our parental generation is initially age-synchronized via alkaline bleach. This very first step of our workflow effectively kills all present bacteria - a necessary step to cause the L1-stage arrest. The L1-stage parental generation and all resultant offspring are subsequently raised on OP50 *E. coli*. Collectively these data suggest that the proposed compounds, excluding cadmium, may be obsogenic in the resultant offspring. Furthermore, because there are no significant differences in body area, these increases in stained area are not simply due to overall increases in offspring size.

Morphological differences in Oil Red-O stained area were also noted. Specifically, in nematodes developmentally exposed to BPA and triclosan the anterior gonad made significantly less of a contribution to the overall percent of body area stained when compared to vehicle exposed controls. These data initially suggested that lipid storage may begin in the anterior gonad and intestine regions. As the depot expands across the nematode, other regions of the body like the head and tail were
thought to begin to make greater contributions to the overall area of the body stained with Oil Red-O. In order to further investigate this phenomenon, average contribution of each segment to the stained area was plotted against the average percentages of the body area stained for each obesogen. It was observed for each obesogen group, with the exception of cigarette smoke extract, that as the percent of body area stained increased, the tail and/or head began to make a larger contribution to overall body area staining as indicated by position beyond vehicle levels (dotted line). Cigarette smoke extract exposed worms only showed visibly increased contributions to stained area from their posterior gonad and intestine region when examined against vehicle control. This analysis however revealed clustering of several teratogens across the four region-specific plots, suggesting potentially different mechanisms of action according to chemical class. For example, cigarette smoke extract and its constituents BAP and nicotine tended to cluster close to each other due to similar percentages of body stained (~34%-35%). But these compounds also clustered together with respect to regional contribution to Oil Red-O stained area as well at levels near those observed in the vehicle group. These data suggest that cigarette smoke extract and its constituents may be working via related mechanisms, and that the lipid storage dynamics may be similar to those in vehicle exposed nematodes. BPA, DES, fenthion, and triclosan however, exhibited a different clustering trend. All three of these had ~34%-37% of their body stained. Secondly, these were grouped above vehicle levels in regional contribution plots for the tail and posterior gonad. In the anterior gonad region plot, all three were clustered below vehicle levels. BPA was not clustered near DES, fenthion, or triclosan in the plot for the head region contribution to stained area. Collectively
these data suggest that the estrogen mimics and certain biocides may be operating via similar mechanisms to alter the distribution of lipids preferentially towards the posterior and tail region of the nematode body. We assume this is not due to overall increases in percent of stained area, as the As(III)O and TBT groups, which exhibited the highest average percentages of body area stained, did not reveal similar trends.

Regarding potential estrogen mimic mechanistic similarity, one such approach to confirm these two agents are acting via similar mechanisms may be to perform a microarray and compare the two profiles of resultant expression data. Since both BPA and DES are estrogen mimics, it may be expected that they have similar effects on gene expression. Additionally, a second more pharmacological approach may also be applied. Mentioned earlier, Hao et al. was able to demonstrate the DES was able to induce adipogenesis in 3T3-L1 cells. However interestingly, they also noted that this effect was mediated by the estrogen receptor (ER), as co-administration with an ER antagonist was sufficient to reduce adipogenesis. Effects were proposed to be due to ER signaling through PPARγ, which was shown to be comparatively reduced in the ER-antagonist group via western blot (Hao et al.; 2012). Interestingly, *C. elegans* also possess a handful of nuclear hormone receptors capable of binding both estrogen and BPA (Mimoto et al.; 2007). Given this information, future work may be directed at employing a similar approach to Hao et al., 2012. Here, administration of an ER antagonist may be able to ameliorate or prevent the obesogenic effects on offspring lipid storage resulting from a developmental exposure to BPA or DES.

The observation of morphological differences is to our knowledge, relatively under-investigated and in need of improved systematic means of evaluation. One RNAi
screen for example has revealed 'disorganized' lipid storage in response to knockdown of sterioidegenic acute regulatory related protein as revealed via Nile Red staining (Ashrafi et al., 2003). However further detailed description or more quantitative analyses are absent. Our novel approach for analyzing lipid storage in the nematode presented here may be used in future, similar studies to better address these potential morphological differences.

Morphological differences in lipid storage may also be evaluated in greater detail in future work using the approach of Hench et al. in 2011. Here, conventional histopathology techniques were used to finely delineate and measure lipid staining in distinct anatomical regions of interest in sagittal nematode cross sections. This made it possible to clearly distinguish and measure differences in Oil Red-O lipid staining between the distal and proximal gonadal regions, as well as the intestine. These cross sections may also be similarly stained for COX, NADH, and SDH to characterize mitochondrial activity levels reveal more insight on the resultant state of offspring energy expenditure (Hench et al., 2011). COX (cytochrome C oxidase), and SDH (succinate dehydrogenase) are two key members of the mitochondrial electron transport chain that uses NADH as currency in the aerobic generation of ATP within this organelle. A potential reduction in the staining density for these subunits, for example, may therefore indicate a reduction in overall mitochondrial activity. If there are concomitant reductions in activity and oxygen consumption, the data would suggest that the 'obese' nematode offspring may be deriving their energy anaerobically.

We have shown here that developmental exposure to obesogens can increase the overall lipid storage and differentially alter depot morphology of the resultant
offspring in *C. elegans*. The outlined approach demonstrated by Hench et al. highlights interesting avenues for future work that may yield greater mechanistic insight to the dynamics of lipid storage in the affected nematode and the related state of energy homeostasis.

### 3.5.2 *C. elegans* Can Be Used to Corroborate and Screen for Putative Obesogens

As a whole, the data presented here further supports those compounds with prior literature-reported obesogenic potential such as CSE, BPA, and DES. However, it also brings to light direct evidence of obesogen inducing effects for other compounds that had only been previously shown to be lipid homeostatic modifiers, such as arsenic(III)oxide and BAP. While the exact mechanistic routes may vary between constituents and categories, several have implicated some form of nuclear hormone signaling.

Regarding cigarette smoke and its constituents, our data corroborate prior experimental work demonstrating the obesogenic potential of CSE and nicotine. Indeed, several studies and even on meta-analysis has shown that epidemiologically, prenatal cigarette smoke exposure predisposes offspring to excess weight gain an obesity (Von Kries et al., 2002; Beyerlin et al., 2011 Oken et al., 2008). Furthermore, several have shown dose-dependency, where children of heavy smokers are at a notably increased risk for obesity than children of light smokers (Harris et al., 2013; Koshy et al., 2010). Experimentally, these findings have been corroborated by Chen et al., who demonstrated that prenatal cigarette smoke exposure in mice was sufficient to induce offspring obesity, even though the exposed offspring consumer fewer calories.
overall (Chen et al., 2011). To our knowledge, similar investigations of these potential effects in *in vivo* invertebrate systems have not performed, making our study unique.

Mechanistically, there are a few means through which CSE and its constituents may be acting to increase offspring lipid storage. Prenatal nicotine exposure alone has been shown to be obesogenic while simultaneously inducing insulin resistance (Gao, et al., 2005). Thus, it may be interesting in future work to investigate the role the equivalent pathway in *C. elegans*. The *C. elegans* insulin receptor homolog daf-2 exerts inhibitory action on the lipogenic NHR daf-16 (Ashrafi et al., 2007). Absence of the obesogenic effects of CSE or nicotine on a daf-16 knockout mutant or RNAi knockdown would therefore suggest these agents may act mechanistically via similar pathways to those noted in mammalian models (Chen et al., 2011, Gao et al., 2005). Regarding BAP specifically, this is the first study to our knowledge to address obesogenic effects *in vivo* from a developmental exposure window. BAP has been shown to in hepatocytes to activate the arylhydrocarbon nuclear receptor (AhR) to mediate thyroid hormone breakdown (Schraplau et al., 2015). Hence, it may be possible that this agent is acting through analogous nuclear hormone related pathways to modulate lipid storage in *C. elegans*. Examination of developmental BAP exposure in conjunction with selective RNAi knockdown of of *C. elegans* homolog AHR-1, for example, may reveal if the obesogenic effects are AhR-mediated (Powell-Coffman, Bradfield, and Wood, 1998).

In addressing the obesogenic potential of heavy metals and metalloids, developmental exposure to cadmium did not induce significant increases in offspring percentages of body staining positive for triglycerides. However, developmental
exposure to arsenic(III)oxide was sufficient to induce significant detectable increases in offspring lipid storage as detectable by percent of body stained. There is little evidence suggesting arsenic to be obesogenic, except for the very recent paper by Rodriguez et al. in 2016, which showed that prenatal in utero exposure to 10ppb and 42.5ppm arsenic in water decreased fecundity and induced moderate obesity in CD-1 mice (Rodriguez et al., 2016). In addition, Kolachi et al. in 2011 did note in one epidemiological study that infants and their diabetic mothers had higher circulating levels of blood cadmium and arsenic compared to infants from nondiabetic mothers (Kolachi et al., 2011). These findings suggest that our obesogenic effects noted with C. elegans may be mediated via the previously mentioned daf-2/daf-16 insulin signaling pathway. Future analysis may be similarly directed at assessing the potential rescue of a developmental arsenic induced ‘obese’ phenotype via daf-16 RNAi knockdown.

To our knowledge this is the first published study to directly addresses the obesogenic potential of the Florida-specific biocide fenthion, and the second to assess the pervasive biocide triclosan (Rodríguez and Sanchez, 2010). The direct effects of triclosan exposure in pregnant rats by Rodriguez and Sanchez in 2010 reveals a potential avenue for further research. Specifically, parental rats were noted to have a dose-dependent reduction in circulating T3 and T4 thyroid hormone levels in response to triclosan treatment throughout gestation and lactation (Rodríguez and Sanchez, 2010). Since impaired thyroid function has been linked to the development of obesity (Boidin, 2010), it is possible that triclosan may have also negatively impacted the developing thyroid of the in utero exposed rat offspring, predisposing them to the excess weight gain they would eventually incur during adolescence (Rodríguez and
Sanchez, 2010). In *C. elegans*, these effects may manifest through alterations in different nuclear hormone receptor homologs and their natural ligands responsible for energy regulation and metabolism.

Regarding BPA and DES, our data appear to support the extensive body of literature detailing the obesogenic potential of these estrogen mimics (vom Saal et al., 1998; Cagen et al., 1999; Howdeshell et al., 1999; Rubin et al., 2001; Miyawaki et al., 2007; Somm et al., 2009; Williams et al., 2014; Riu et al., 2014; Newbold et al., 2005; Hao et al., 2012). Several of these studies have highlighted modulation of PPARγ in response to treatment with these agents, highlighting this nuclear hormone receptor as a potential mechanistic player in the excess production of lipids and weight gain (Somm et al., 2009; Riu et al., 2014; Hao et al., 2012). Interestingly, the biocide TBT has also been shown to alter nuclear hormone activity of PPARγ in vitro and in vivo (Grun et al., 2006; Riu et al., 2014). Our data also support the prior literature highlighting the obesogenic effects of this biocide. The role of nuclear hormone receptor signaling in the development of these lipogenic phenotypes noted in prior work highlights a potential mechanistic pathway for the obesogenic effects observed within our study. Indeed, while prior works have highlighted putative *C. elegans* homologs to protective PPARα like NHR-49 (Pathare et al., 2012; VanGilst et al., 2005), none as of yet have reported a homolog to the obesity-promoting PPARγ (Ashrafi, 2007).

Taken together, the above findings highlight the important role of nuclear hormone signaling in the development of excess lipid accumulation in response to obesogen exposures. Therefore, it is possible that NHR signaling pathways may be similarly mediating lipid storage in our obesogen-exposed nematodes. *C. elegans* have
284 nuclear hormone receptors (Antebi, 2006). Hence the search for the appropriate signaling homologs to human NHRs may be best aided by bioinformatics techniques that capitalize on conserved sequence-structure-function relationships.

3.5.3 Obesogens May Alter Energy Balance in C. elegans

While there were no significant increases in pharyngeal pumping rate in the obesogen exposed offspring, those exposed to CSE or As(III)O exhibit significantly reduced pumping rates. These exposed offspring took on average 29-39 fewer pharyngeal pumps per minute when compared to vehicle exposed controls. It would be expected then, that these offspring also have a corresponding significant reduction in activity level, however this was not the case.

While offspring developmentally exposed to each of the obesogens displayed on average lower body thrashing rates than vehicle control exposed offspring, these differences were not statistically significant. This may be due to a confounding reduction in assay sensitivity resulting from a change in physical platform. Because the nematodes were moved from solid NGM media to a liquid drop of M9 buffer, they may have encountered additional stress that obscured our ability to detect significant differences in basal activity- In other words, they were compelled to move by the new environment. In the future, three methods could be used to enhance the assessment of energy expenditure in nematode offspring. The first would be the use of a Clark-type oxygen electrode to measure changes in oxygen consumption. However this assay, conventionally takes place in a liquid platform and provides only information on aerobic respiration based energy expenditure. A second method may be to employ an infrared body thrashing assay amenable for multi-well plates (Simonetta and Golembek, 2007).
Such an assay infers body thrashing rate from the number of infrared beam breaks, and the use of multi-well plates may allow for a notable increase in sample size which would add sufficient statistical power towards this aspect of the study. However this assay too, still takes place on a liquid platform.

In order to assess the physical behavior of the nematode offspring on a solid platform, it would be preferable to implement a worm tracking software designed for use with solid NGM plates. The worm tracking software would be most useful when used in conjunction with a Clark-type oxygen sensing electrode. Husson et al. described in great detail how historically, these programs were developed to bring experimental consistency and enhance quantitative assessment of behavioral characteristics that were previously only characterized by qualitative descriptions. These programs, depending on the complexity, may track from 1-120 nematodes on a single plate. Typically, they operate by defining the center of mass or ‘centroid’ for the nematode. The change in centroid position throughout the movie is measured as a function of time elapsed to yield the velocity of a nematode. One drawback of these programs is that they are typically cost prohibitive to set up (Husson, 2012).

Secondly, it is a literature highlighted issue that alternative methods for assessing energy intake aside from pharyngeal pumping are needed (Al-Anzi and Zinn, 2011). Because of this, one ongoing project in the lab is directed at generating a fluorescing strain of *E. coli* OP50. Theoretically, *C. elegans* could be allowed to ingest this glowing strain of *E. coli* for a specific period of time, after which, the fluorescence signal in its pharynx or intestine could be used to infer the volume of bacteria ingested. The generation and optimization of such a tool would be tremendously useful in
conjunction with the pharyngeal pumping assay, as it would allow researchers to more finely characterize feeding rate and volume, respectively.

The results of this portion of the behavioral studies are not conclusive. However, the fact that there were significant reductions in pharyngeal pumping rates for two of the offspring groups highlights the potential for changes in energy balance warranting greater and more complex elucidation. With tools such as the worm tracker and oxygen electrode, these new avenues for future research may be able to more finely detect and define homeostatic changes in energy expenditure within the resultant nematode offspring.

3.5.4 Obesogen Screen Conclusions

The results of this study establish a concrete, *in vivo*, screening platform for developmental obesogens. This platform is able to detect changes in overall area of the Oil Red-O stained lipid storage across the body as well as changes in defined regional lipid depots. Furthermore, while the behavioral assays were not conclusive, they did reveal that developmental exposures may have lasting changes on offspring behavior into adulthood. Future work may be best directed at further defining the energy state of obesogen exposed offspring through assessment of mitochondrial function, oxygen consumption, and innate activity levels in their natural environment. Additionally, nuclear hormone receptors appear to be a shared signaling mechanism through which these agents may program these alterations in lipid storage and energy homeostasis. The NHR family of proteins in *C. elegans* therefore presents itself as an attractive candidate for future mechanistic studies involving obesogen signaling pathways.
3.6 References


3.7 Tables and Figures

Figure 3.1: An illustrated schematic of the performed obesogen screen workflow. (1) the age-synchronized parental generation are grown up on media containing the obesogen or control for 57 hours until they are gravid adults, at which point (2) they are moved to untreated media to lay embryo offspring for three hours. (3) These offspring are metabolically phenotyped in adulthood to assess lipid content and energy balance (energy intake vs. energy expenditure).
Table 3.1: Mean body area, stained area, and percent of body stained for obesogen screen offspring. The mean body area (pixels$^2$), stained area (pixels$^2$), and percent of body stained are shown for nematode offspring in all groups. Total sample size per group summed across 5 replicates ranged from 143-165 nematodes/group. (*) indicates p<0.05.

Average of F1 Body Area, Stained Area, and Percent of Body Stained

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean body area (pixels$^2$ ± SEM)</th>
<th>Mean stained area (pixels$^2$ ± SEM)</th>
<th>Mean % body stained (% ± SEM)</th>
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<tr>
<td>0.01% DMSO Vehicle</td>
<td>157</td>
<td>219930 ± 2802.</td>
<td>67702 ± 3861</td>
<td>29.12 ± 1.52</td>
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<tr>
<td>10μM BPA</td>
<td>149</td>
<td>216754 ± 3196</td>
<td>83183 ± 4346*</td>
<td>36.32 ± 1.70</td>
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<tr>
<td>10μM DES</td>
<td>165</td>
<td>226243 ± 3020</td>
<td>84180 ± 3996*</td>
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</tr>
<tr>
<td>0.1μM Triclosan</td>
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<td>228268 ± 2849</td>
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<td>37.17 ± 1.45</td>
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<td>0.1μM TBT</td>
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<td>221311 ± 2451</td>
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<td>0.1μM Fenthion</td>
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<td>218826 ± 3024</td>
<td>79286 ± 5000*</td>
<td>34.40 ± 2.12</td>
</tr>
<tr>
<td>1.0μM CSE</td>
<td>158</td>
<td>220248 ± 3031</td>
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<td>34.38 ± 1.61</td>
</tr>
<tr>
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<td>225034 ± 13557</td>
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<td>0.5μM BAP</td>
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<tr>
<td>0.5μM Cd</td>
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<td>209125 ± 2559</td>
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<tr>
<td>0.5μM As(III)O</td>
<td>163</td>
<td>210696 ± 2746</td>
<td>82367 ± 3756*</td>
<td>37.61 ± 1.59</td>
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</table>
Figure 3.2: Obesogens affect stained area but not body area. (A) Shows the average body areas (pixels$^2$ + SEM) for nematode offspring in all groups. (B) Shows the average stained areas (pixels$^2$ + SEM) for nematode offspring in all groups. Total sample size per group summed across 5 replicates ranged from 143-165 nematodes/group. *=p<0.05.
Figure 3.3: Obesogen exposed offspring have increased percentages of body area Oil Red-O stained. Shows the average percent of body stained (% ± SEM) for nematode offspring in all groups. Total sample size per group summed across 5 replicates ranged from 143-165 nematodes/group. *=p<0.05.
Figure 3.4: Representative images of Oil Red-O stained obesogen exposed offspring. Shows representative Oil Red-O stained obesogen-exposed nematode offspring under the vehicle control. Size bar =100μm.
Figure 3.5: Illustration of the anatomical regions assessed. Depicted are the regions of the *C. elegans* body individually analyzed for stained area.

Table 3.2: Average contribution of regions to overall stained area in obesogen exposed offspring. Displays the average % contribution (%± SEM) of each body region to the overall stained area in the *C. elegans* body across groups. Total sample size per group summed across 5 replicates ranged from 143-165 nematodes/group. *=p<0.05.

| Average Percent Contribution of Body Segment Region to Total F1 Stained Area |
|-----------------------------|--------|-----------------|-----------------|-----------------|-----------------|
|                             | N     | Tail region (% ± SEM) | Posterior gonad region (% ± SEM) | Anterior gonad region (% ± SEM) | Head region (% ± SEM) |
| 0.01% DMSO Vehicle         | 157   | 0.29 ± 0.07        | 47.69 ± 0.78          | 50.77 ± 0.76          | 1.25 ± 0.19      |
| 10µM BPA                   | 149   | 0.39 ± 0.08        | 49.63 ± 0.86          | 47.92 ± 0.81*         | 2.06 ± 0.27*     |
| 10µM DES                   | 165   | 0.43 ± 0.14        | 49.73 ± 0.79          | 48.96 ± 0.78          | 0.87 ± 0.14      |
| 0.1µM Triclosan            | 160   | 0.45 ± 0.10        | 50.10 ± 0.99*         | 48.39 ± 0.95*         | 1.06 ± 0.15      |
| 0.1µM TBT                  | 151   | 0.17 ± 0.06        | 47.12 ± 0.71          | 50.96 ± 0.79          | 1.74 ± 0.27      |
| 0.1µM Fenthion             | 152   | 0.35 ± 0.09        | 50.01 ± 0.88*         | 48.63 ± 0.86          | 1.01 ± 0.17      |
| 1.0µM CSE                  | 158   | 0.27 ± 0.07        | 48.28 ± 0.93          | 50.34 ± 0.89          | 1.11 ± 0.14      |
| 0.5µM Nicotine             | 152   | 0.27 ± 0.07        | 46.79 ± 0.77          | 51.27 ± 0.77          | 1.66 ± 0.23      |
| 0.5µM BAP                  | 153   | 0.43 ± 0.11        | 47.52 ± 0.75          | 50.67 ± 0.75          | 1.31 ± 0.22      |
| 0.5µM Cd                   | 143   | 0.30 ± 0.07        | 46.88 ± 0.87          | 51.40 ± 0.85          | 1.42 ± 0.22      |
| 0.5µM As(III)O             | 163   | 0.30 ± 0.08        | 49.09 ± 0.76          | 49.16 ± 0.81          | 1.45 ± 0.36      |
Figure 3.6. The regional percent contribution in obesogen exposed offspring as a fraction of all stained area. The average % contribution of each body region to the overall stained area in the *C. elegans* body is graphed across groups. Changes in the contributions of these regions to overall stained area are better visualized on a region-specific basis (Figures 3.6 and 3.7)
Figure 3.7: Obesogens visibly alter percent contribution by tail and posterior gonad region. (A) Shows the average percent contribution (+ SEM) of solely the tail region to the average stained area of the *C. elegans* body across all groups. (B) Shows the average percent contribution (+ SEM) of solely the posterior gonad region to the average stained area of the *C. elegans* body across all groups. Total sample size per group summed across 5 replicates ranged from 143-165 nematodes/group.
Figure 3.8: Obesogens alter percent contribution by head and anterior gonad regions. (A) Shows the average percent contribution (± SEM) of solely the anterior gonad region to the average stained area of the C. elegans body across all groups. (B) Shows the average percent contribution (± SEM) of solely the head region to the average stained area of the C. elegans body across all groups. Total sample size per group summed across 5 replicates ranged from 143-165 nematodes/group. *=p<0.05.
Figure 3.9: Select obesogens show similar patterns in regional contributions and percent of body stained. Shown are plots of average body segment contributions to the stained area against the average percent of body stained for each obesogen. Colored ovals indicate clustering events of interest.
Table 3.3: CSE and As(III)O exposed offspring have reduced pharyngeal pumping rates. Displayed are the mean pharyngeal pumps per minute (± SEM) in nematode offspring across all groups. The total sample size per group summed across five replicated ranged from 41-50 nematodes/group. * = p<0.05

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<td>0.5μM As(III)O</td>
<td>50</td>
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Figure 3.10: CSE and As(III)O exposed offspring have reduced pharyngeal pumping rates. Graphically shown are the mean pharyngeal pumps per minute (± SEM) in nematode offspring across all groups. The total sample size per group summed across five replicated ranged from 41-50 nematodes/group. * = p<0.05
Table 3.4: Obesogen exposed offspring do not have altered body thrashing rates. Displays the mean body thrashes per minute (+ SEM) in nematode offspring across all groups. The total sample size per group summed across five replicated ranged from 41-50 nematodes/group.

<table>
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<tr>
<td>0.5μM As(III)O</td>
<td>46</td>
<td>187.57 ± 6.04</td>
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</tbody>
</table>

Figure 3.11: Obesogen exposed offspring do not have altered body thrashing rates. Graphs the mean body thrashes per minute (+ SEM) in nematode offspring across all groups. The total sample size per group summed across five replicated ranged from 41-50 nematodes/group.
CHAPTER FOUR

FUNCTIONAL BIOINFORMATICS ANALYSIS OF PUTATIVE OBESOGENIC PATHWAYS

4.1 Bioinformatics as a Tool to Find Homologs in Phylogenetically Distant Species

A phylogenetic analysis is aimed to identify the evolutionary relationships between a set of protein and/or nucleic acid sequences. Typically, this is achieved with bioinformatics tools such as a multiple sequence alignment (MSA) of the set of sequences. Here, the similarity between the set of sequences is examined as a whole to find regions of optimum sequence similarity. The exact type of alignment, the method, and the evolutionary substitution assumptions used may vary depending on the choice of molecule(s) for analysis, phylogenetic relatedness of the sequences, and overarching goal of the alignment.

There are two major types of sequence alignment, local and global. A more common local alignment method, known as the Smith-Waterman alignment, finds stretches of the most conserved regions between sequences, and was originally designed to find gene regulatory elements in DNA (Smith and Waterman, 1981; Mount, 2004). However if the end-goal is to assess overall relatedness between protein sequences from different species, a global alignment, such as a Needleman-Wunch alignment, may be a better choice. The global method will align full-length sequences
from end to end and attempting to computationally find the arrangement that produces the greatest similarity among sequences (Needleman and Wunch, 1970; Mount, 2004).

Progressive and iterative methods are two common approaches for physically building a multiple sequence alignment. The progressive method starts with aligning the most similar sequences, and then continues to align lesser-related sequences to the prior alignment (Hogeweg and Hesper, 1984). Unfortunately, potential errors in the initial alignment are propagated and amplified throughout these later steps of the multiple sequence alignment, making the end result less reliable (Mount, 2004). To resolve this, iterative-based methods repeatedly will align small subsets of sequences to each other, and then back to the overall multiple sequence alignment. This process thereby helps to assess the overall alignment and make the appropriate adjustments for its refinement (Edgar, 2004).

Assumptions on the rates of substitution may be specific to the choice of molecule for analysis. For nucleotide sequences, for example, the Jukes Cantor model assumes the rates of substitution for A, C, T, and G are the same between sequences (Jukes and Cantor, 1969). The Kimura correction however takes into consideration the frequencies of transitions and transversion events (Kimura 1980). For protein sequences, the Poisson model assumes the rates of substitution for all amino acids are the same (Nei and Kumar, 2000). The PAM (point accepted mutation) and related JTT (Jones, Taylor, and Thorton) models however assume substitution rates that have been derived from previously examined from analysis of sequences with high (>85%) identity and/or known structure (Schwarts and Dayhoff, 1979; Jones, Taylor, and Thorton, 1992). Different assumptions on the rate of substitution will differentially affect the
similarity score of the multiple sequence alignment, which is used to generate the evolutionary distances between sequences. The evolutionary distance is what ultimately dictates connectedness and branch lengths between sequences on a phylogenetic tree (Mount, 2004).

There are three major strategies to build a phylogenetic tree, and their applicability depends on the number and variation between sequences in the dataset. Maximum parsimony is one main method that first examines all of the possible trees that could explain the observed variation. Then it joins together sequences in a manner requiring the fewest number of steps, and therefore tree nodes, to explain this observed variation. For this reason, it is also referred to as the ‘minimum evolution’ method (Fitch, 1971). While maximum parsimony attempts to generate the optimal tree, it is computationally intensive and best suited to examine a small set of closely related sequences (Mount, 2004). The distance method is more applicable for investigating a larger set of sequences. In this method, the sequence similarity from the multiple sequence alignment is used to generate a distance matrix. The distance data is then used to compute branch lengths and connections between sequences in the phylogenetic tree (Fitch and Margoliash, 1967). One drawback however is that it is not as sensitive as maximum parsimony (Mount, 2004). A third major method, maximum likelihood, is suitable for larger numbers of less related sequences. This method like maximum parsimony, will first examine all of the possible trees to explain the observed variation. However, it will then use a specific evolutionary substitution assumption to calculate the likelihood a certain tree, and therefore evolutionary chain of events, that might have occurred. It ultimately generates the “most likely” phylogenetic tree to
explain the relationship between sequences. (Felsenstein, 1981). The ability to examine and choose different evolutionary assumption models is an advantage. However, like maximum parsimony, maximum likelihood also adds a great degree of computational complexity (Whelan and Goldman, 2001).

Once an appropriate set of methods has been chosen, the analysis may be bootstrapped to increase confidence in the observed tree. Nearest neighbors with shorter branch lengths represent closer evolutionary lineages, and are therefore the best-suited candidates for functional evaluation. Phylogenetic analyses have proven to be a useful tool in several fields ranging from conservation biology to pharmacy (Tennant, 2003). In this interdisciplinary lipid metabolism study, we employ phylogenetic analyses to find close *C. elegans* nuclear hormone receptor (NHR) relatives to human PPARγ, PPARα, and PPARδ. After selecting our mostly likely candidates, we are assessing their functional role on triglyceride storage in the nematode via RNAi knockdown techniques.

4.2 RNAi Background and Utility in Obesity Research

RNA interference (RNAi) is a tool used in *C. elegans* work where exogenous application of dsRNA by injection, soaking, or feeding suppress the translation of specific transcripts depending on sequence similarity. The feeding method is the most cost effective and the most amendable for screening projects (Ahringer, 2006). In this method, bacteria carry engineered plasmids to transcribe both sense and anti-sense fragments of a specific *C. elegans* cDNA that anneal together to form the dsRNA. Upon ingestion, the bacteria are lysed and the dsRNA is released into the gut for absorption (Timmons & Fire, 1998). Once transported inside the *C. elegans* cells, the dsRNA
induces recruitment of specific proteins and formation of a RISC complex that splices the dsRNA sequence into smaller segments to ultimately form an antisense-guide siRNA. The guide siRNA hybridize to mRNA transcripts of the gene of interest, targeting them for degradation. Without the mRNA transcripts, there is no corresponding translation to protein. This process effectively and efficiently disrupts expression of genes in the nematode (Figure 4.1).

Early work with this technology by Timmons and Fire in 1998, noted low-penetrating or weak changes in phenotype (Timmons and Fire, 1998). However, the same group soon after found that *E. coli* strains lacking in RNaseIII enzymes like HT115, accumulated the most dsRNA, greatly increasing the efficacy of gene suppression (Timmons, Court, and Fire 2001). The group performed a handful of tests to evaluate the strength of the suppression via the feeding methodology, and full interference was defined as >95% of the cells in a nematode showing an absence of signal. When *myo-3::gfp* nematodes were fed HT115 containing a plasmid directed against GFP, more than 98% of the animals exhibited complete interference. This was in stark comparison to other strains of bacteria used such as BL21 and W3110, where only about 15% of the nematodes showed complete interference. To ensure this suppression performed equally as well on *C. elegans* genes, RNAi via feeding was also performed testing the different bacterial strains containing plasmids directed against *unc-22*. Effective suppression of *unc-22* yields an ‘uncoordinated’ or twitching phenotype. Again, HT115 RNaseIII deficient bacteria proved to be the most effective by comparison, with 100% of animals showing the twitching phenotype compared to only 0-26% seen in other strains. Authors highlight that the RNAi administered via
feeding specifically, has been shown to be more effective of a means of RNAi delivery as resistance had been demonstrated in response to the microinjection method. Furthermore, they conclude that the RNAi-mediated gene suppression via the fed HT115 RNAseIII deficient *E. coli* produced resultant phenotypes that were comparable to those seen in loss-of-function mutants (Timmons, Court, and Fire 2001). The ability of this method to reliably and specifically silence gene expression in the nematode has since been consistently demonstrated, and is a key advantageous feature for this molecular tool in *C. elegans*.

RNAi screens have been used in prior studies to help elucidate pathways and identify novel regulators implicated in the lipid storage process. Recently, one group has generated a transgenic worm expressing a GFP fusion to a *Drosophila* perilipin (PLIN) protein shown to reside specifically in lipids. The worms express this PLIN::GFP which is visible as ‘rings’ around their lipid droplets. Interestingly, an RNAi screen of 1600 genes verified some pathways, but also revealed some new, unevaluated genes involved in increased lipid storage visualized via increased PLIN::GFP signal. For example, RNAi daf-2 knockdown resulted in an increased ring size PLIN::GFP expressing lipid droplets, a result confirmed with conventional Nile Red staining. However, RNAi suppression of 9 previously uncharacterized genes were also revealed to be lipogenic as visualized by an increase in PLIN::GFP signal, similarly verified by Nile Red (Liu et al., 2014). Additionally, other prior work screening 16,757 genes with RNAi has demonstrated 305 genes in *C. elegans* that reduce lipid storage and 112 genes that increase lipid storage. Interestingly, RNAi of members from the nuclear hormone receptor gene family were shown to result in both increased and reduced fat
phenotypes. Suppression of genes involved in sterol-related pathways resulted in abnormal distributions of fat content, further highlighting the importance of gene regulation of not only total fat levels, but the storage depots (Ashrafi et al., 2003). The highlighted studies demonstrate the utility of RNAi in conjunction with other tools such as staining and fluorescent markers, to reveal novel regulators of fat storage in Caenorhabditis elegans.

4.3 Hypothesis

It was hypothesized that phylogenetic analysis of C. elegans NHRs to human PPARs will provide functional gene candidates that underlie signaling for the lipogenic action of BPA. If there is a decrease in lipid storage with corresponding negative shifts in energy balance, these NHRs may be functional homologs of hPPARγ. If there is an increase in lipid storage with a corresponding positive shift in energy balance, these NHRs may be functional homologs of hPPARα or hPPARδ.

4.4 Methods

4.4.1 Multiple Sequence Alignment and Phylogenetic Tree Assembly

First, a simple homology search was performed for Caenorhabditis elegans protein sequence homologs to the amino acids sequences for human PPARγ (gil13432234), PPARδ (gil417522), and PPARα (gil50348666) using NCBI’s Basic Local Alignment Search Tool (BLAST). The resultant list of C. elegans proteins was pruned by removing first those sequences with an E-value greater than 0.001. The resultant list of sequences was downloaded. Secondly, duplicate sequences and pseudogenes were manually searched for and removed. This produced a set of 257 sequences. The pruned set of sequences was saved as a ‘.txt’ file and imported into the MEGA version 6
(Tamura, Stecher, Peterson, Filipski, and Kumar 2013). MUSCLE (Multiple Sequence Alignment by Log-Expectation) was used to perform a multiple sequence alignment with the following parameters: gap open penalty = -2.9, gap extend penalty = 0, hydrophobicity multiplier = 1.2, clustering method (all iterations): UPGMB. The MSA was subsequently saved as a ‘.mas’ file and exported as a ‘.meg’ file (Edgar, 2004).

The ‘.meg’ file used in MEGA6 was used to assemble three initial trees using the maximum likelihood method. Each tree was generated using one of the following evolutionary substitution models - Poisson, JTT, or WAG (Whelan and Goldman, 2001). Trees were assembled using the following parameters: rates among sites = gamma distributed (G), number of discrete categories = 5, gaps/missing data treatment = complete deletion, ML heuristic method = nearest neighbor interchange (NNI), branch swap filter = very strong. These initial three trees may be found in Appendix A. Once the three initial trees were made, subtrees were selected from each and the closest C. elegans sequences to the cluster of hPPARγ, hPPARα, and hPPARδ, were used to generate a refined list of 13 sequences. The amino acid sequences from this refined list were then re-aligned by MUSCLE and imported to MEGA6 to generate a second set of phylogenetic trees using the same maximum likelihood approach, evolutionary substitution models, and parameters. Because our refined list was an order of magnitude smaller than our initial set of sequences, we added a parameter, bootstrapping each individual phylogenetic tree with 500 replications. This allowed us to pick nearest neighbors with increased confidence of evolutionary relatedness. The top 3 candidates- NHR-85, NHR-23, and SEX-1, were chosen for downstream RNAi knockdown studies.
4.4.2 RNAi Strains

W05B5.3 (NHR-85), C01H6.5 (NHR-23), and F44A6.2 (SEX-1) plasmid-containing strains of *E. coli* HT115 were ordered from GE Healthcare Dharmacon Inc. (#RCE1181, RCE1182) from the library constructed by Marc Vidal (Rual et al., 2004). The inserts directed against these NHRs span the length of their respective genes. Figure 4.1 depicts the graphic map of the empty vector pL4440 as generated by SerialCloner 2.6.1 (Frank Perez, 2013). HT115 bacteria containing empty vector pL4440 was provided as a generous gift by Dr. Sandy Westerheide. The plasmid inserts were verified by sequencing and checked for potential off-target mRNAs using a nucleotide blast against the *C. elegans* genome sequence. This revealed that NHR-85, SEX-1, and NHR-23 insert sequences uniquely matched the respective genes and have no similarity to any off-target genomic regions or cDNAs in *C. elegans*. Sequencing for the NHR-23 directed plasmid failed, and no concrete match was found via blast. Similarly, sequencing and nucleotide blast analysis of the empty vector confirmed that it did not match any *C. elegans* gene sequence.

4.4.3 *C. elegans* Strain and Colony Maintenance

*C. elegans* were maintained as described in 2.3.1.

4.4.4 Developmental synchronization

*C. elegans* age-synchronization was performed as described in 2.3.2.

4.4.5 Chemicals

Bisphenol-A containing NGM plates were made as described in 2.3.3.
4.4.6 Exposure Design and RNAi Knockdown

On the first day of the experiment, glycerol stocks of the RNAi bacterial strains and empty vector were streaked onto LB agar plates containing 50μg/ml ampicillin and incubated overnight at 37°C for approximately 16 hours. On this day, several mixed-stage plates of nematodes age-synchronized by alkaline bleach preparation as described in 2.3.2. One set of 100mm petri plates containing either 0.01% DMSO vehicle or 100μM BPA in 0.01% DMSO in NGM lite were seeded with 100μL of 1X OP50 and incubated for 16 hours overnight at 25°C.

One day two of the experiment, 500-1000 of the hatched L1 C. elegans were placed onto the vehicle or BPA containing NGM plates, which were then let to dry and incubate at 25°C for 57 hours. On this day, NGM plates for RNAi were made as follows: 1.5g sodium chloride, 1.25g bacto-peptone, and 10g agar per 500mL of ultrapure water were placed into a glass bottle with and autoclaved for 30 minutes. After autoclaving, the bottle was placed into a large enough beaker that contained a small layer of water, which was then placed on a hot plate. The contents of the bottle were gently stirred for approximately 60 minutes until the temperature read 55°C, and the following were added aseptically per 500mL of NGM: 0.5mL 5mg/ml cholesterol in 100% ethanol, 0.5mL 1M calcium chloride, 0.5mL 1M magnesium sulfate, 12.5mL 1M potassium phosphate monobasic, 0.5mL 1M IPTG (Isopropyl β-D-1-thiogalactopyranoside), and 0.25mL 100mg/ml ampicillin. The resultant NGM was poured into 60mL petri plates at a volume of 6mL. These plates were left to dry overnight at room temperature. After RNAi plates were made, liquid cultures were also started for each of the RNAi strains and empty vector streaked the day prior. A colony
was picked and used to inoculate 100-120mL of LB supplemented with 100μg/ml ampicillin and 12.5ug/mL tetracycline. These cultures were allowed to grow at 37°C and 220 RPM for 16 hours overnight in incubator bacterial shaker (Excella E24 Incubator Shaker Series, New Brunswick Scientific, NJ, USA).

On the third day of the experiment, the bacterial cultures were checked for their OD$_{600}$, and were subsequently spun down and concentrated to approximately 10X. 120μL of the concentrated RNAi or bacteria with empty vector was used to seed RNAi plates, which were then let to dry before incubation at 25°C until use.

When the parental *C. elegans* had aged to 57 hours by the fourth day of the experiment, they were gently washed with M9 buffer 5 times, spun at 1000 RPM and aspirated between washes. The washed parent nematodes from the BPA group were then plated onto the three RNAi containing plates alongside empty vector containing plates. The washed parent nematodes from the vehicle exposure group were placed only onto empty vector containing plates. These parent nematodes were allowed to lay eggs, at which point they were individually removed from the plates. These offspring were allowed to grow on the untreated NGM plates with RNAi or empty vector containing HT115 for approximately 60 hours, at which point they were assessed for metabolic and behavioral phenotypes as described in chapter 3.3. For an illustration of the work flow, see Figure 4.2. All experiments were doubly coded. With respect to Figure 4.2, the first set of codes were made on the plates intended for egg-laying by BPA-exposed parents (prior to step 2). The second set of codes were made on all plates after egg-laying by both vehicle and BPA-exposed parents (after step 2). Data was decoded upon collection and submission to the coder.
4.4.7 Oil Red-O Lipid Staining

*C. elegans* were stained as described in 3.3.5.

4.4.8 Image Analysis

Image analysis of *C. elegans* was performed as described in 3.3.6.

4.4.9 *C. elegans* Collection and Homogenization

The Triglyceride Colorimetric Assay Kit used was purchased from Cayman Chemical Company (10010303, Ann Arbor, MI). Manufacturer’s instructions were followed to reconstitute the included standard diluent assay reagent to 1X, which as stored at 4°C and brought to room temperature before each use. Nematode samples were collected in sets of 500 nematodes in 50μL of 1X standard diluent and stored at -20°C until homogenization. For the bead homogenization method, 0.5mg glass beads 0.5mm in size (GB05, Next Advance, Averill Park, NY) were added to the collected nematode sample and mixed by vortexer on the highest setting 3X in 10-15 minute intervals for an approximate total of 30-45 minutes. Samples were kept on ice between cycles of vortex processing. Sample homogenization was visually confirmed under a stereomicroscope. Once the sample was homogenized, a 100μL or 200μL pipette man was used to recover the sample from the tube and transfer it into a separate, sterile tube for the TAG assay. The number of intact nematodes and the percent recovery per tube was recorded. One average, there were 13.48 (+5.64 SD) nematodes lost during the process of homogenizing the sample through transfer to the bead-containing tube or failure to break down. This yielded an average of 486.24 ± 5.19 nematodes per processed sample. The average percent recovery was 66.95% (+ 5.27% SD). Once the samples were homogenized, they were centrifuged at 10,000g for 10 minutes at 4°C
as per manufacturer’s instructions and frozen (-20°C) until used in the colorimetric coupled enzymatic triglyceride assay.

4.4.10 Colorimetric Coupled Enzymatic Triglyceride Assay

The Triglyceride Colorimetric Assay Kit used was purchased from Cayman Chemical Company (10010303, Ann Arbor, MI). A standard curve was made fresh using the 1X standard diluent and a provided triglyceride standard at 200mg/dL according to manufacturer’s instructions with one exception being the inclusion of an additional dilution. The standard concentrations were ultimately: 200, 100, 50, 25, 12.5, 6.25, 3.125, 1.5625, and 0 mg/dL. The sodium phosphate assay buffer provided with the kit was diluted with 16mL of ultrapure water. This buffer was used to make the triglyceride enzyme mixture. The triglyceride enzyme mixture was reconstituted with 1mL of ultrapure water, after which it was transferred to a 15mL conical vial wrapped in aluminum foil. To this, 14mL of the sodium phosphate assay buffer was added. The solution was gently mixed by inversion.

To perform the assay, 10µL of each standard and each sample was pipetted in technical duplicate into a 96-well microtiter plate. To begin the reaction, 150µL of the triglyceride enzyme solution was added to each well of the plate. The plate was covered with optical tape and left to incubate at room temperature for 15 minutes. The absorbance via plate reader was read at 540nm.

4.4.11 Bradford Protein Quantification Assay

‘Coomassie Plus- The Better Bradford Reagent’ (#23238, Pierce Biotechnology, Rockford, IL) was used as per manufacturer’s guidelines. Briefly, manufacturer’s instructions were followed to generate a set of standards by diluting bovine serum
albumin (2mg/mL dissolved in 0.9% saline with 0.05% sodium azide) with 0.9% saline to: 1500, 1000, 750, 500, 250, 125, 25, and 0 μg/mL (Pierce Biotechnology, Rockford, IL). 0.9% saline was used to make 1:2 dilutions of each of the nematode sample. 10μL of the diluted sample was added to 300μL of room temperature Coomassie reagent, gently mixed, and left to incubate at room temperature for 10 minutes. Manufacturer’s instructions were followed for the adaptation of the Bradford Assay for use with the Nanodrop 2000/2000c (Thermo Fisher Scientific, Wilmington, DE). For each standard and sample, the assay was performed in technical quintuplicate.

4.4.12 Pharyngeal Pumping Assay

Pharyngeal pumping rate was assessed as in 3.3.10.

4.4.13 Body Thrashing Assay

Body thrashing rate was assessed as in 3.3.11.

4.4.14 Statistical Analysis

Statistical Package for the Social Sciences (IBM SPSS Version 23) was used to perform descriptive and inferential statistical analyses on the body area, stained area, percent of body stained, and behavioral data. Data were analyzed via One-way ANOVAs followed by LSD post-hoc analysis.

Microsoft Excel was used for the generation of the linear standard curve generated in the Colorimetric Coupled Triglyceride Assay. Manufacturer’s instructions were also followed to generate a 4-parameter polynomial equation of best fit for the standard curve in the Bradford Assay using Microsoft Excel (Pierce Biotechnology, Rockford, IL). Statistical Package for the Social Sciences (IBM SPSS Version 23) was used to perform descriptive and inferential statistical analyses on the resulting
concentrations of triglycerides and proteins, and generate corresponding figures. Data were analyzed via one-way ANOVAs followed by LSD post-hoc analysis.

4.5 Results

4.5.1 Phylogenetic Analysis Reveals Candidate hPPAR Homologs for Functional Analysis

After the first three preliminary global MSAs of the set of 257 sequences, the sequences appearing in the subtree containing hPPARγ, hPPARδ, and hPPARα exhibited significant overlap in their neighboring proteins (Appendix A). These overlapping neighboring proteins were then used to generate a second set of 13 sequences that underwent a second multiple sequence alignment to hPPARγ, hPPARδ, and hPPARα. After realignment, the same 3 evolutionary models were used to compute phylogenetic trees with bootstrapping. Observing the bootstrapped trees, NHR-85, NHR-23, and SEX-1 were the most commonly aligned neighbors to hPPARγ, hPPARδ, and hPPARα (Figure 4.2). To our knowledge, none of these 3 candidates had prior experimental literature detailing any effects on lipid storage. However, NHR-23 was known to be expressed during embryogenesis, and SEX-1 knockouts were known to increase embryonic lethality (Kostrouchova et al., 1998). These presented an additional concern for our experimental design because lethality in our offspring generation would preclude the experiments on offspring lipid storage. To circumvent a potential lethal phenotype, the RNAi was administered post-egg laying by the parental generation to affect offspring development post-gastrulation (Figure 4.1) (Sulston et al., 1983).
4.5.2 RNAi Suppression of NHRs After BPA Exposure Alters Oil Red-O Stained Area and Triglyceride and Protein Content

The total sample size per group across 4 experimental replicates for the whole body stain analysis was 122-136 nematodes/groups. A total number of 656 nematodes were assessed in this experiment. Significant effects were seen with respect to overall body size of the offspring generation (ANOVA F (4,651) = 9.365, p < 0.001). Nematodes exposed developmentally to BPA who were raised on vector-only control HT115 exhibited significantly reduced body areas that were approximately 5.79% smaller (11485 pixels\(^2\)) than vehicle exposed offspring raised on vector-only control. Additionally, nematodes exposed to BPA who were raised on any of the RNAi-containing strains exhibited significantly increased average body areas that were 5.25-9.28% greater (10418-18388 pixels\(^2\)) compared to BPA exposed offspring raised on vector-only control (Figure 4.3A).

Significant effects were also seen with respect to Oil Red-O stained area of the offspring generation (ANOVA F (4,651) = 44.635, p < 0.001). Here, offspring exposed developmentally to BPA raised on any of the RNAi-containing strains exhibited significantly increased Oil Red-O stained areas when compared to BPA-exposed offspring raised on vector-only control. These RNAi-treated offspring Oil Red-O stained areas were 137-213% greater (20614-43878 pixels\(^2\)) than the stained areas of vector-only control offspring (Figure 4.3B). There was no significant difference in stained area between vehicle-exposed and BPA-exposed offspring raised on vector-only containing control (p>0.05).
The percent of body area Oil Red-O stained was significantly altered in the nematode offspring as well (ANOVA F(4,651) = 55.036, p < 0.001). Offspring developmentally exposed to BPA raised on vector-only control had significantly reduced percentages area of body stained compared to vehicle-exposed offspring raised on vector-only control. All BPA-exposed offspring raised on an RNAi-containing HT115 showed significantly increased percentages of body Oil Red-O stained ranging from approximately 8%-21% more than BPA-exposed offspring fed vector-only (Figure 4.4). Analysis of the body segments revealed visible morphological differences in lipid storage of NHR suppressed offspring compared to those BPA-exposed nematodes raised on empty vector only, described in further detail below (Figure 4.6).

The sample size per group across 4 experimental replicates for the body segment analysis was 122-136 nematodes/groups. A total number of 656 nematodes were assessed in this experiment. Specifically, nhr-23, nhr-85, and sex-1 knockdown groups exhibited significantly increased contributions to their overall stained area by their tail region (ANOVA F(4, 651) = 12.998, p < 0.001) (Figure 4.7A). It appeared that in nhr-23 and sex-1 knockdown offspring, that the contribution by the posterior gonad region was increased and decreased respectively, compared to those BPA-exposed nematodes raised on empty vector only (Figure 4.7B). These trends however only approached statistical significance (p = 0.075 and 0.052, respectively). Significantly increased contributions by the head region were also noted in the nhr-23 and sex-1 knockdown groups (ANOVA F(4, 651) = 16.248, p < 0.001) (Figure 4.8B). These two groups however exhibited significantly reduced contributions from their anterior gonad region (ANOVA F(4, 651) = 4.793, p < 0.005) (Figure 4.8A).
When the percent of body stained was plotted against the average regional contribution (Figure 4.9), it appeared that RNAi knockdown groups exhibiting higher overall percentages of body stained also tended to have greater contributions to the Oil Red-O stained area from their head and tail regions. These knockdown groups also appeared to have reduced contributions from their anterior gonad region, falling behind levels seen for BPA exposed offspring raised on vector only. No apparent trends were seen in the plot for the posterior gonad region.

BPA-exposed offspring raised on RNAi-containing HT115 also appeared to have increased triglycerides and protein levels than BPA-exposed offspring raised on vector-only. Specifically, all RNAi-exposed offspring groups exhibited between 0.36-0.66 more ng triglycerides/mL per nematode (Figure 4.5A), but this was not detected to be statistically significant. RNAi-exposed offspring from the NHR-23 knockdown group exhibited the highest overall protein levels, having nearly 2.5 times as much μg protein/mL per nematode than BPA exposed offspring raised on vector-only control (Figure 4.5B). Lastly, when triglycerides were normalized to protein, only offspring from the SEX-1 and NHR-85 knockdown groups showed significant increases compared to BPA-exposed offspring fed vector-only controls. Specifically, these RNAi-exposed offspring exhibited between approximately 2.1 and 2.5 times as much ng triglycerides/μg protein when compared to their empty-vector control counterparts. Offspring from the NHR-23 knockdown group however exhibited nearly a 30% reduction in the amount of ng triglycerides/μg protein (Figure 4.6, Table 4.2).
4.5.3 RNAi Suppression of NHRs After BPA Exposure Does Not Significantly Alter Energy Balance

For the pharyngeal pumping assay, the total sample size per group summed across three experimental replicates ranged from 29-30 nematodes/group. A total of 148 nematodes were assessed in the pharyngeal pumping assay. For the body thrashing assay, the total sample size per group summed across three experimental replicates ranged from 21-29 nematodes/group. A total of 129 nematodes were assessed in the body thrashing assay. No significant effects were noted in the body thrashing or pharyngeal pumping assays (Figure 4.13, Figure 4.14). Offspring from the nhr-23 knockdown group appeared to have the highest overall pharyngeal pumping rates compared to their vector-only counterparts, taking on average approximately 5 more pharyngeal pumps per minute; the effect however, was not statistically significant. Offspring from the nhr-85 knockdown group appeared to have the lowest overall pharyngeal pumping rates compared to their vector-only counterparts, taking on average approximately 16 fewer pharyngeal pumps per minute; the effect however, only approached statistical significance (p = 0.12).

4.6 Discussion

The data presented here highlight the very interesting, novel reported phenotypes of NHR-23, NHR-85, and SEX-1 knockdown on lipid storage in C. elegans. Knockdown via RNAi of these NHRs tends to increase lipid storage as detectable via Oil Red-O. These data suggest at first glance that these NHRs may function more like antilipogenic PPARα than PPARγ, however these unanticipated findings are still new. Likewise, the results of the enzymatic triglyceride assay more closely mirror the staining
data when observed solely as ng triglycerides/mL per nematode. Here, knockdown of all three NHRs produced visible increases in the amounts of triglycerides present in the sample and nematodes. These data corroborate the Oil Red-O staining data, where knockdown of all three NHRs significantly increased the percent of body stained. Significant effects however were not determined via one-way ANOVA in the enzymatic triglyceride assay and more replicates may be necessary to increase power. Another possibility these results suggest is that staining with Oil Red-O and analyzing the resultant images may a more sensitive method to detect subtle significant differences in finer changes to lipid storage in the nematode. The enzymatic triglyceride assay data was nonetheless interesting, and when coupled with protein data, revealed other intriguing changes in the resultant nematode body composition.

NHR-23 for example, is typically expressed in the hypodermis of the nematode and is required for proper molting during larval growth (Kostrouchova et al., 1998). In particular, we noticed that nhr-23 knockdown nematodes had higher overall triglycerides (ng triglycerides/mL/nematode), but also the highest overall levels of protein. Thus, when we corrected the triglyceride for protein levels, nhr-23 knockdown exhibited the lowest levels of ng triglycerides/μg protein. Regardless, NHR-23 holds some promise in the realm of lipid storage regulation. MacNeil et al. found in 2013 that RNAi directed against nhr-23 reduced expression of Pacdh-1::GFP (MacNeil et al., 2013). Acyl CoA dehydrogenase (acdh) is an important enzyme involved in the beta-oxidation of fatty acids. Hence, it may be possible that knockdown of nhr-23 in our study had inhibitory effects on potentially downstream pathways involved in lipid breakdown. Because cell number is fixed in the nematode (Sulston et al., 1983), it may be possible that nhr-23
knockdown may also have a function the regulation of cellular size as well. Additionally, while the trend was not significant, our behavioral data revealed *nhr-23* knockdown nematodes exhibited the highest overall pharyngeal pumping rates. While we cannot draw conclusions on whether or not this increase in feeding rate is causal for the increase in body size or required by it, these results highlight a new and previously unreported potential behavioral phenotype associated with knockdown of this NHR.

It should be noted that *nhr-23* is also expressed during embryogenesis and RNAi knockdown via microinjection has been demonstrated to result in L3 to L4-stage larval arrest (Kostrouchova et al., 1998). One study has overcome this obstacle by reducing the concentration of HT115 delivered to their animals (MacNeil et al., 2013). However, we did not observe any effects of larval arrest in our nematode offspring. This suggests that we may have overcome the molting defect by administering our RNAi via feeding after early embryonic development. A second, speculative, hypothesis is that the developmental exposure to BPA may somehow be protective against *nhr-23* RNAi induced molting defects, although work testing this same knockdown on a BPA-free background would need to be done to rule out this possible interaction.

Knockdown of *nhr-85* had similar, however less pronounced effects. NHR-85 is also expressed in the hypodermis, as well as the vulva and rectal tissue. It is similarly thought to be involved in molting because of its resemblance to 20-hydroxyecdysone, a protein involved in insect molting. Knockdown of this NHR however does not induce molting defects, but rather reduces the number of progeny by about one-third (Gissandaner, 2004). Limited experimental information defining the role of *nhr-85* is available. However, one study has found that *nhr-85* was upregulated in response to
dietary restriction (DR) conditions (Ludewig, 2005). Normally, DR conditions increase nematode tolerance to external stressors like heat (Houthoofd, Johnson, and Vanfleteren 2005). Interestingly in Ludewig et al.’s study, nhr-85 mutant worms were found to be increasingly susceptible to heat stress induced death under DR conditions, suggesting that this NHR is required for stress resistance (Ludewig, 2005). In our present study, when triglycerides were normalized for protein in our nhr-85 knockdown group of offspring samples, there was still a noticeable increase compared to BPA-treated offspring raised on vector-only control. Our data in conjunction with the literature reveal the effects of this NHR may be far-reaching across the nematode body, with implications ranging from stress resistance, metabolism, and reproduction. Our findings regarding lipid staining and storage are, to our knowledge, also the first reported for RNAi knockdown of this NHR. These data taken together highlight a new and exciting avenue warranting further mechanistic investigation.

The knockdown of sex-1 had different, specific effects on lipid and protein levels. Offspring exposed developmentally to BPA who were raised on sex-1 RNAi bacteria had visibly similar levels of μg protein/mL per nematode when compared to BPA exposed offspring raised on vector only control. The offspring in the sex-1 RNAi group additionally had one of the highest levels of ng triglycerides/mL per nematode. Hence, when triglycerides were normalized to protein, offspring in the sex-1 group exhibited the highest overall increase in relative lipid storage compared to their vector-only raised control counterparts. These data highlight that contrary to the predictive information in the Uniprot database, this NHR is likely to function more as an anti-lipogenic PPARα than the lipogenesis-promoting PPARγ. Sex-1 is a NHR responsible for xol-1
transcriptional repression in *C. elegans*, a gene required for the generation of male-sex in the nematode (Carmi, Kopczynshi, and Meyer, 1998, Carmi and Meyer, 1999). To our knowledge, our results are the first to highlight this new and interesting role of *sex-1* on lipid homeostasis in the nematode.

In summary, the staining data was a more sensitive tool for the detection of changes in overall lipid storage in the nematode offspring. The enzymatic triglyceride assay is still necessary, as it revealed macromolecular changes in body composition that further cemented the PPARα-like properties of some NHRs, like *sex-1*, while calling into question others like *nhr-23*. However the enzymatic triglyceride assay data in conjunction with the results of the Bradford assay highlight, future work should also be done to examine these lipid-modifying effects without a teratogen exposure. Should we not observe similar effects resulting from RNAi knockdown, this would present the intriguing possibility that these NHRs work to suppress a specifically BPA-programmed increase in fat storage.

Additionally, we note in these experiments that developmental exposure to BPA resulted in the unanticipated slight reduction of lipid storage in adulthood. This may be due to a handful of reasons. The first is that the OP50 bacteria on the exposure plate may have metabolized BPA, rendering it inactive (Volkel, 2002). Additionally, in order to perform RNAi experiments, NGM plates must be made containing ampicillin and IPTG. These two exogenously applied agents are not present within the standard *C. elegans* NGM lite used within our obesogen screen. Additionally, NGM lite used for the obesogen screen has slightly less salt (-1g/L) and more bactopeptone (+1.5g/L) than NGM prepared for RNAi plates. It is possible that these may add a confounding factor
that obscures our ability to detect BPA-induced effects within this study. Indeed, prior work has found that common experimental agents used for C. elegans experiments like FUdR (5-fluoro-2'-deoxyuridine) can confound the very results they are used to test (Feldman, Kosolapov, and Ben-Zvi, 2014). Lastly, RNAi experiments are typically carried out using the HT115 strain of E. coli engineered to be RNaseIII null. It is possible then that the effects seen in our two groups were due to the use of a different postnatal diet in this study (HT115) compared to the prior obesogen screen from chapter 3 (OP50). Indeed, several studies have noted opposing phenotypes with respect to lifespan depending on the diet administered (Brooks, Liang, and Watts, 2009; Soukas et al., 2009; MacNeil et al., 2013; Mizunuma et al., 2014; Pang and Curran, 2014). Pertinently, Brooks et al. noted in 2009 that nematodes raised on OP50 did have higher triglyceride/protein ratios (Brooks, Liang, and Watts, 2009). Because of these differences, Xiao et al. in 2015 developed an OP50 strain that was able to knock down the expression of target genes with equivalent efficacy to the traditional HT115 strain (Xiao et al., 2015). The adaptation of the RNAi library into the OP50 strain should be a tremendous asset to the elucidation of diet-induced effects on offspring phenotype. In particular, using such a tool future work may be aimed at assessing the effects of the postnatal nutritional environment resulting from prenatal exposures in C. elegans. Nonetheless, the effects found within this study highlight the new and exciting potential of our candidate NHRs to modify lipid storage and deposition within the nematode.

Lastly, we acknowledge that the use of a candidate approach may still not yield sufficient insight into the mechanistic signaling pathways through which obesogens act in C. elegans. In such a situation, it may be best to use a type of forward genetics
approach. In this approach, mutagenesis in the form of UV light or ethyl methanesulfate (EMS), for example, may be used to isolate exposed offspring who do not show the resultant phenotype (Kutscher and Shaham, 2014). Once the selected offspring are isolated, the responsible mutation may be mapped and the affected gene more thoroughly evaluated for function. For this process however, certain considerations must be made. First, both of our specific assays for lipid distribution and quantification are terminal assays, resulting in the killing of the nematode during sample processing. Hence, in order to assess lipid content while maintaining viability (required for isolation), it may be necessary to use a less specific technique such as Nile Red (O'Rourke et al., 2009). However, permitting greater accessibility it should be possible that the use of the previously mentioned CARS microscopy could be used to isolate live C. elegans possessing the desired phenotype (Jeong Lee et al., 2015). Such an approach would provide greater insight into those genes ultimately responsible for the development of increased lipid storage in the resultant obesogen-exposed offspring in C. elegans.

In conclusion, the data presented here highlights the lipid-altering effects in C. elegans of select NHRs bearing sequence similarity to the human PPAR family of proteins. Future work may be expanded using our workflow to functionally test other homologs from our phylogenetic analysis. Such information may provide greater insight into the dynamic between lipid storage and energy balance in the nematode.

4.7 References


4.8 Tables and Figures

Figure 4.1: Illustration of RNAi signaling events involved in disruption of gene expression in *C. elegans*.
Global Alignment: Aligns sequences from end to end to find the optimum alignment

Figure 4.2: A depiction of a global sequence alignment.

Figure 4.3. Graphic map of pL4440 empty vector.
Figure 4.4: An illustrated schematic of the performed RNAi experimental workflow. (1) The age-synchronized parental generation are grown up on media containing the BPA or 0.01% DMSO vehicle seeded with 1X OP50 E. coli for 57 hours until they are gravid adults, at which point (2) The vehicle treated parents are washed thoroughly and moved to an untreated plate containing 10X HT115 E. coli containing vector only. BPA treated parents are washed thoroughly and moved to untreated plates seeded with 10X HT115 E. coli containing vector only or a plasmid directed against one of the three candidate NHRs. The parental generation is allowed to lay embryo offspring for three hours. (3) These offspring are metabolically phenotyped in adulthood to assess lipid content and energy balance (energy intake vs. energy expenditure).
Figure 4.5: Phylogenetic analysis reveals candidate hPPARγ, hPPARδ, and hPPARα homologs for functional analysis. Shown are the three bootstrapped phylogenetic trees of the refined list of sequences generated by different evolutionary assumptions. Each has a very similar set of neighbors to hPPARγ, hPPARδ, and hPPARα.
Table 4.1: Mean body area, stained area, and percent of body area stained in RNAi treated offspring. Displays the mean body area (pixels\(^2\)), stained area (pixels\(^2\)), and percent of body stained for nematode offspring in all groups. Total sample size per group summed across 4 replicates ranged from 122-136 nematodes/group. * = p < 0.01 compared to Vehicle + Empty vector. † = p < 0.01 compared to BPA + Empty vector.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Mean body area (pixels(^2) ± SEM)</th>
<th>Mean stained area (pixels(^2) ± SEM)</th>
<th>Mean % body stained (% ± SEM)</th>
</tr>
</thead>
<tbody>
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<td>209734 ± 204880</td>
<td>27171 ± 1651</td>
<td>12.75 ± 8.46</td>
</tr>
<tr>
<td>BPA + Empty vector</td>
<td>135</td>
<td>196248 ± 3061</td>
<td>20615 ± 1533*</td>
<td>9.68 ± 8.30*</td>
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<tr>
<td>BPA + nhr-23 RNAi</td>
<td>136</td>
<td>220306 ± 2801†</td>
<td>48875 ± 2704†</td>
<td>21.33 ± 12.18†</td>
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<tr>
<td>BPA + nhr-85 RNAi</td>
<td>132</td>
<td>216636 ± 3113†</td>
<td>40265 ± 2916†</td>
<td>17.43 ± 12.18†</td>
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<tr>
<td>BPA + sex-1 RNAi</td>
<td>122</td>
<td>203866 ± 2478†</td>
<td>64493 ± 3621†</td>
<td>30.02 ± 17.49†</td>
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</table>
Figure 4.6. RNAi suppression of NHRs alters body area and stained area. (A) Shows the average body area (pixels$^2$ ± SEM) for nematode offspring in all groups. (B) Shows the average stained areas (pixels$^2$ ± SEM) for nematode offspring in all groups. Total sample size per group summed across all 4 replicates ranged from 122-136 nematodes/group. * = p < 0.01 compared to Vehicle + Empty vector. † = p < 0.01 compared to BPA + Empty vector.
Figure 4.7: RNAi suppression of NHRs increases percent of body stained. Shown are the average percent of body stained (% + SEM) for nematode offspring in all groups. Total sample size per group summed across all four replicates ranged from 122-136 nematodes/group. * = p < 0.01 compared to Vehicle + Empty vector. † = p < 0.01 compared to BPA + Empty vector.
Figure 4.8: Illustration of the anatomical regions assessed. Depicted are the regions of the *C. elegans* body individually analyzed for stained area.

Table 4.2: Average contribution of regions to overall stained area in RNAi treated offspring. Displays the average % contribution (%± SEM) of each body region to the overall stained area in the *C. elegans* body across groups. Total sample size per group summed across 4 replicates ranged from 122-136 nematodes/group. * = p < 0.01 compared to Vehicle + Empty vector. † = p < 0.01 compared to BPA + Empty vector.

<table>
<thead>
<tr>
<th></th>
<th>N</th>
<th>Tail region (%± SEM)</th>
<th>Posterior gonad region (%± SEM)</th>
<th>Anterior gonad region (%± SEM)</th>
<th>Head region (%± SEM)</th>
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<tr>
<td>Vehicle + Empty vector</td>
<td>131</td>
<td>0.18 ± 0.12</td>
<td>46.12 ± 1.08</td>
<td>51.24 ± 1.09</td>
<td>2.46 ± 0.60</td>
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<td>135</td>
<td>0.10 ± 0.07</td>
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<td>51.56 ± 1.48</td>
<td>1.02 ± 0.37*</td>
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<td>BPA + nhr-23 RNAi</td>
<td>136</td>
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<td>50.35 ± 1.14</td>
<td>45.16 ± 1.07†</td>
<td>3.20 ± 0.47†</td>
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<td>BPA + sex-1 RNAi</td>
<td>122</td>
<td>1.18 ± 0.20†</td>
<td>43.92 ± 1.24</td>
<td>48.53 ± 1.18</td>
<td>6.37 ± 0.63†</td>
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Figure 4.9: The regional percent contribution in RNAi treated offspring as a fraction of all stained area. The average % contribution of each body region to the overall stained area in the C. elegans body was graphed across groups. Changes in the contributions of these regions to overall stained area are better visualized on a region-specific basis (Figures 4.7 and 4.8)
Figure 4.10: RNAi suppression of NHRs alters percent contribution by tail and posterior gonad regions. (A) Shows the average percent contribution (+ SEM) of solely the tail region to the average stained area of the *C. elegans* body across all groups. (B) Shows the average percent contribution (+ SEM) of solely the posterior gonad region to the average stained area of the *C. elegans* body across all groups. Total sample size per group summed across 4 replicates ranged from 122-136 nematodes/group. † = p < 0.01 compared to BPA + Empty vector.
Figure 4.11: RNAi suppression of NHRs alters percent contribution by head and anterior gonad regions. (A) Shows the average percent contribution (± SEM) of solely the anterior gonad region to the average stained area of the C. elegans body across all groups. (B) Shows the average percent contribution (± SEM) of solely the head region to the average stained area of the C. elegans body across all groups. Total sample size per group summed across 4 replicates ranged from 122-136 nematodes/group. * = p < 0.01 compared to Vehicle + Empty vector. † = p < 0.01 compared to BPA + Empty vector.
Figure 4.12: RNAi suppression of NHRs alters patterns in regional contributions and percent of body stained. Shows plots of average body segment contributions to the stained area against the average percent of body stained for each group.
Figure 4.13. Representative images of Oil Red-O stained RNAi treated offspring. Shown are representative Oil Red-O stained nematode offspring from each group reflective of the average % of body stained. Size bar = 100μm.
Figure 4.14: RNAi suppression of NHRs alters levels of triglycerides and protein.  
(A) Graphs the average ng triglycerides (TAG)/mL per nematode from nematode samples across all groups.  (B) Graphs the average μg protein/mL per nematode from samples across all groups.  Total number of nematodes per sample ranged was on average $486 \pm 5$.  Data are reflective of averages taken from 5 experimental replicates.
Figure 4.15: RNAi suppression NHRs alters ratio of triglycerides to protein. Graphs the average ng triglycerides (TAG)/μg protein across all groups. Data are reflective of averages taken from 5 experimental replicates.
Table 4.3: RNAi suppression of NHRs alters levels of triglycerides and protein. Shows the average levels of triglycerides and protein per mL per nematode, as well as the normalized triglyceride/protein ratios across all treatment groups.

<table>
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<tr>
<th>Treatment</th>
<th>ng triglycerides/mL per nematode (± SEM)</th>
<th>μg protein/mL per nematode (± SEM)</th>
<th>ng triglycerides/μg protein (± SEM)</th>
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<td>0.58 ± 0.13</td>
<td>0.84 ± 0.07</td>
<td>0.69 ± 0.15</td>
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<td>BPA + Empty vector</td>
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<td>0.60 ± 0.17</td>
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<td>BPA + nhr-23 RNAi</td>
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<td>2.56 ± 0.59</td>
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<td>BPA + sex-1 RNAi</td>
<td>1.10 ± 0.51</td>
<td>1.14 ± 0.38</td>
<td>1.53 ± 0.63</td>
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Table 4.4: RNAi suppression of NHRs does not significantly affect pharyngeal pumping rate. Displayed are the mean pharyngeal pumps per minute (± SEM) in nematode offspring across all groups. The total sample size per group summed across 4 replicates ranged from 29-30 nematodes/group.

<table>
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<tr>
<th></th>
<th>N</th>
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<tr>
<td>Vehicle + empty vector</td>
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<td>179.06 ± 4.58</td>
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<td>BPA + empty vector</td>
<td>30</td>
<td>188.87 ± 4.74</td>
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<tr>
<td>BPA + nhr-23 RNAi</td>
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<td>193.87 ± 3.60</td>
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<tr>
<td>BPA + nhr-85 RNAi</td>
<td>30</td>
<td>172.65 ± 12.76</td>
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<tr>
<td>BPA + sex-1 RNAi</td>
<td>29</td>
<td>180.47 ± 7.50</td>
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Figure 4.16: RNAi suppression of NHRs does not significantly affect pharyngeal pumping rate. Graphically depicts the mean pharyngeal pumps per minute (± SEM) in nematode offspring across all groups. The total sample size per group summed across 4 replicates ranged from 29-30 nematodes/group.
Table 4.5: RNAi suppression of NHRs does not significantly affect body thrashing rate. Displays the mean body thrashes per minute ($\pm$ SEM) in nematode offspring across all groups. The total sample size per group summed across 4 replicates ranged from 21-29 nematodes/group.

<table>
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<th></th>
<th>N</th>
<th>TPM ($\pm$ SEM)</th>
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<tr>
<td>Vehicle + empty vector</td>
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<td>182.57 $\pm$ 5.81</td>
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<td>BPA + empty vector</td>
<td>23</td>
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<td>BPA + nhr-23 RNAi</td>
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<td>180.64 $\pm$ 5.99</td>
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<td>BPA + nhr-85 RNAi</td>
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<td>186.75 $\pm$ 4.21</td>
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<tr>
<td>BPA + sex-1 RNAi</td>
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<td>182.28 $\pm$ 6.68</td>
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Figure 4.17: RNAi suppression of NHRs does not significantly affect body thrashing rate. Graphically depicts the mean body thrashes per minute ($\pm$ SEM) in nematode offspring across all groups. The total sample size per group summed across 4 replicates ranged from 21-29 nematodes/group.
5.1 Adverse Reproductive Effects of Developmental Teratogen Exposure

The data presented here highlight the tested compounds (with the exception of nicotine) as overtly reprotoxic following chronic, larval parental exposure. Estrogen mimics tended to reduce the average number of eggs laid on day 1 post-exposure, suggesting a reduction in the number of germ cells available for fertilization. All biocides at some dose shifted the day of maximal egg-laying, suggesting a developmental delay. The effects on gonad development may be lasting, because there was similarly a reduction in the number of eggs laid on day 1 post-exposure. Only TBT however, induced significant reductions in embryonic viability. Cigarette smoke extract and nicotine did not negatively affect reproductive development or embryonic viability, contrary to prior literature (Sobinoff et al., 2013). However, BAP induced reprotoxic effects identical to those seen in the biocide group. Lastly, the heavy metals and metalloids significantly affected all four of the categories for reproductive fitness assessed, shifting the day of maximal egg-laying while concomitantly reducing the number of eggs laid, hatched larvae, and embryonic viability. These effects are identical to those seen by TBT, suggesting that metals specifically may have lasting embryotoxic consequences.
These results hold tremendous implications for the study of low dose teratogen exposures in fields of reproductive biology and environmental health. First, we have demonstrated that the noxious effects of chronic, low dose exposure to these compounds may have lasting effects into adulthood that negatively affect reproductive fitness and embryo viability, even after exposure to the agent ceases. Secondly, we have set up a workflow to test such effects consistently, maintaining an isolated exposure that encompasses gonadal development in the nematode. Together this work further establishes *C. elegans* as an ideal model organism to screen *in vivo* for toxic substances. Here, multiple potential teratogens can be assessed in detail at several doses to adequately cover the scope of exposures. Reductions in egg-laying may indicate a depletion of the germ cell pool. This may be further evaluated by directly dissecting out the *C. elegans* gonad and DAPI-staining it to assess the number of germ cells in the various stages of development (Allard and Coláiacovo, 2010). Reductions in embryonic viability may indicate lasting negative effects on germ cell function, such that fertilized oocytes are unable to appropriately undergo embryogenesis. Because the nematode and its eggs are transparent, it may be possible then to observe the cell stage at which appropriate embryogenesis ceases (Sulston et al., 1983). Further work may then be directed at addressing the roles of proteins known to be essential during embryogenesis that may be affected as a result of the exposure (Zipperlen et al., 2001; Fernandez et al., 2005).

The platform presented here provides a basis for examining lasting changes on the adult reproductive system resulting from chronic exposure to environmental
teratogens. The approach may be applied to characterize alterations in reproductive fitness and aid in the elucidation of causal pathways for reprotoxicity.

5.2 Adverse Metabolic Effects of Developmental Teratogen Exposure

The data presented here also highlight the tested compounds (with the exception of cadmium) as obesogenic, even at very low doses when exposed during early development. These data concerning BPA, DES, TBT, and triclosan, support similar findings from several prior in vivo studies (Holloway 2005 et al., Rodríguez and Sanchez, 2010; vom Saal et al., 1998; Somm et al., 2009; Riu et al., 2014; Newbold et al., 2005). Agents that significantly increased the percentage of body area stained with Oil Red-O dye did so without altering overall body area. Furthermore, both BPA and triclosan exposed offspring exhibited morphological changes in the distribution of their lipid stained areas. Regarding BPA specifically, as the anterior gonad region began to play less of a role, the head region began to make a significantly greater contribution to the overall Oil Red-O stained area. When regional contributions were plotted against percent of body area stained, clustering events revealed interesting trends. Estrogen mimics (BPA and DES) for example, showed consistent clustering patterns across three of the regional plots. This suggested a similar mechanism of action between the two teratogens. Taken together, these data highlight C. elegans as a useful model organism to detect changes in lipid storage induced by developmental teratogen exposures. The platform holds promise for the field of environmental health, which has recently begun to focus on this emerging field of developmental obesogens (Thayer, Heindel, & Bucher, 2012).
We also present here that once an obesogenic agent has been identified, a variety of approaches may be taken to further evaluate offspring phenotype and mechanism of action. Our behavioral data were inconclusive in providing insight into the directionality of energy balance changes. However, the fact that two of the groups did show significantly reduced pharyngeal pumping rates highlights the intriguing possibility for lasting changes in offspring behavior as a result of developmental exposures. Future work could be aimed at addressing oxygen consumption using a Clark-type electrode or nematode velocity using a worm tracker.

Bioinformatic and functional analysis of nuclear hormone receptors is also an appealing avenue for future mechanistic research. While our data regarding the role of nhr-23, nhr-85, and sex-1 in BPA-mediated obesogenic signaling did not reveal a functional PPARγ, significant increases in lipid storage in knockdown groups reflective of PPARα activity were nonetheless observed. These data indicate that RNAi techniques may be successfully applied to this workflow to reveal changes in lipid storage resulting from candidate suppression. Future work may be directed at assessing more distantly related nuclear hormone receptors from our phylogenetic analysis.

Collectively, the data presented here highlights the need for more stringent and expanded screening of consumer goods prior to their market dissemination. Indeed, the substitute for BPA, bisphenol-S (BPS), has been recently found to have similar lipogenic and PPARγ modulating effects (Boucher, Ahmed, and Atlas, 2015). Hopefully, the following platform may be applied towards the investigation and removal of these new
compounds (Bilbrey, 2014). To this end, we may more adequately protect the metabolic fate and health of future generations.

5.3 Future Directions: A High-Throughput Adaptation

One of the primary future directions for this work would be a high-throughput adaptation. Development of an equivalent high-throughput screen (HTS) would be most beneficial for detection of obesogenic compounds prior to their entry into the market, but may also be amendable for large RNAi or drug screens as lipid modifiers. Liu et al. highlight that fixation protocols are less suitable for large scale screens, and vital-dye protocols using Nile Red tend to non-specifically label lysosome-related organelles (Liu et al., 2014; O’Rourke et al., 2009). In order to establish a lipid-droplet specific platform to expedite the assessment of *C. elegans* fat storage, Liu et al. generated a transgenic nematode expressing a GFP fusion to *Drosophila* perilipin (PLIN) under the control of promoters specific to the nematode intestine, hypodermis, and muscle. PLIN proteins are known to specifically reside in the phospholipid monolayer surrounding lipid droplets. The resultant nematodes expressed green fluorescing lipid droplets, which could be individually counted and measured for size. Using this transgenic nematode, they were able to experimentally confirm the specificity of the GFP::PLIN using a different lipid dye, LipidTOX (Liu et al., 2014). Liu et al. developed a novel *in vivo* tool that given the proper physical and technical platform, could easily be adapted for the high-throughput screening of putative developmental obesogens.

First, the physical platform would require the ability to expose the parental generation while providing a means to separate them from their offspring later on. This may be accomplished using a multi-well plate insert. Because *C. elegans* eggs are
approximately 50μm long and 30μm wide (Begasse and Hyman, 2011), a 50μm pore-size insert may be most suitable. Because this experiment would take place in a multi-well plate, liquid S-media (common for liquid culture of C. elegans) would be used instead of NGM. The plate would need to be placed on a gentle, shaking rotator in order to provide proper aeration to the nematode culture. With the insert in the multi-well plate, exposure and control media may be loaded in, and a developmentally synchronized P0 generation may be added at the L1-stage. These parental nematodes may be allowed to grow until the desired age in adulthood, at which point they should be too large to pass through the insert.

These parental nematodes may then be washed with fresh S-media and transferred to a separate multi-well plate containing untreated S-media. The parental generation may be allowed to lay eggs here for the desired time, and the eggs should pass through the 50μm insert into the untreated media. After the egg-laying period, the insert can be removed along with the parent generation. All that should be remaining in the wells at this point are embryos. The multi-well plate can be placed back on the rotating shaker and the offspring can be allowed to hatch and grow until the desired time-point. If we are using Liu’s transgenic GFP::PLIN expressing strain (Liu et al., 2014), changes in fluorescence signals may be used to detect differences in lipid storage.

An enhanced technical platform may further expedite the generation of multiple data end-points, including altered fluorescence, simultaneously. The COPAS™ FP-250 large particle flow cytometer with the LP Sampler™ (Union Biometrica, Inc., Holliston, MA) was designed for use with small, living organisms in multi-well plates. Using this
tool, additional measurements can be made such as counts of the number of nematode offspring that would provide information on fecundity from the parental exposure. Reduced live offspring for example, may indicate that fewer eggs were laid or that fewer of these eggs hatched. Such data would suggest reprotoxic effects, serving as detectable phenotypes for future research. Additionally, we may also be able to measure the fluorescence of individual nematode offspring as well as regional fluorescence localization to head, center, or tail regions. These data would provide information not only on the body-wide lipid storage in the nematode, but also potential differences in regional lipid storage depots. Lastly, this cytometer is capable of maintaining viable nematodes after assessment, such that follow-up studies on behavior to assess energy balance are possible (COPAS FP™ Instruments, Brochure). Figure 5.1 illustrates a proposed, potential workflow for the aforementioned high-throughput adaptation.

Ultimately, the proposed high-throughput platform would generate data that would add a new facet of obesogen-altered offspring metabolic health to the volume of ToxCast (Toxicity Forecaster) information already generated from existing high-throughout toxicity screens. These types of screening studies have taken place since 2008, and have been encouraged through the NIH-EPA collaboration Tox21 (NIH News Release, 2008). Tox21, standing for Toxicology in the 21st Century, is a cooperative effort between the two institutions to promote the development of in vitro toxicity screens and screens using simple model organisms to identify and assess compounds that pose a hazard to human health (NTP, 2013). The intention of the project was to minimize reliance on more time-consuming mammalian animal studies (NIH News
In this dissertation we demonstrate that the Fetal Origins of Health and Disease Hypothesis may be modeled in *Caenorhabditis elegans*. New technology may now be adapted to develop similarly modeled high-throughput screens to address this hypothesis. Collectively, the findings modeled through use of this nematode and the ideas presented for future work presented here make significant contributions to the study and protection of offspring health from its earliest possible developmental stages. The establishment of these types of screens is paramount for the protection of future generations’ public health and crucial for the prevention of disease.

### 5.4 References


1) A 50µm pore size multi-well plate insert is placed into a multi-well plate containing exposure media.

2) Alkaline bleach age-synchronized P0 are raised on insert in exposure media in multi-well plate.

3) P0 are moved on the insert to a separate multi-well plate containing untreated media to lay eggs for 3 hours. Eggs, ~50µm long and 30µm wide, fall through the insert. The insert and P0 are removed after the egg-laying period.

4) Egg-laying synchronized F1 hatch and grow until adulthood in the untreated media of the multi-well plate. Once adults, they may be assessed for lipid storage.

5) A top-down illustration of an individual well shows what would be an expected view of the resultant F1 transgenic for perilipin-GFP under a fluorescent microscope. The COPAS FP-250 (Union Biometrica) may then be used to detect and measure the distribution of perilipin-GFP coated lipid droplets in individual nematodes. These data would provide information on the overall lipid storage as well as depot morphology.

Figure 5.1: Annotated illustration of a potential high-throughput adaptation for a developmental obesogen screen.
WAG initial tree
Poisson initial tree
JTT initial tree