5-2016

Environmental Toxicants and Unsaturated Fatty Acids Alter Toxicant Sensitivity and Allocation of Polar Lipids in Daphnia Magna

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ENVIRONMENTAL TOXICANTS AND UNSATURATED FATTY ACIDS ALTER TOXICANT SENSITIVITY AND ALLOCATION OF POLAR LIPIDS IN DAPHNIA MAGNA

A Dissertation
Presented to
the Graduate School of
Clemson University

In Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy
Environmental Toxicology

by
Namrata Sengupta
May 2016

Accepted by:
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Dr. Peter van den Hurk
ABSTRACT

*Daphnia* is a commonly used test organism in toxicology, ecology, and evolution. HR96 is a CAR/PXR/VDR ortholog in invertebrates, and a promiscuous endo- and xenobiotic nuclear receptor involved in acclimation to toxicants. *Daphnia* HR96 is activated by chemicals such as atrazine and linoleic acid (LA) (n-6 fatty acid), and inhibited by triclosan and docosahexaenoic acid (DHA) (n-3 fatty acid). HR96 is also a key regulator of Magro, Niemann Pick genes, and other genes involved in cholesterol and triacylglycerol uptake, metabolism, and transport. We hypothesized that *Daphnia magna* exposed to HR96 activators will increase their lipid resources and in turn either develop a tolerance towards starvation or demonstrate increased reproduction relative to untreated animals or animals exposed to HR96 inhibitors, and this phenotype would be associated with the retention or production of certain lipids. Exposure of late adolescent daphnids to fatty acids (DHA and LA) reduced the starvation survival of their offspring. However, DHA exposed juvenile daphnids typically survived following reproduction during starvation indicating greater nutrient stores and overall health of these animals than the other treated groups. Interestingly, the HR96 inhibitor, triclosan, significantly increased offspring survival compared to all other groups except the untreated daphnids. Additional experiments demonstrated that juveniles exposed directly to triclosan also showed increased survival but did not mature and therefore never reproduced. Lipidomic analysis comparing starved adults and neonates showed that neonates have relatively more sphingomyelins and relatively less phosphatidylcholine than adults. The HR96
inhibitors, DHA and triclosan significantly increased sphingomyelins, and DHA down-regulated magro and a sphingomyelinase; two HR96-regulated genes crucial in producing sphingosine from the sphingomyelins. It is interesting to speculate that a drop in sphingosine, a key developmental lipid, may be associated with greater longevity and slow/no development. In conclusion, fatty acids and toxicants can cause sublethal effects by altering the resource allocation of lipids and in turn perturbing starvation survival, reproduction, and juvenile development.
DEDICATION

This thesis is dedicated to Dr. Stephen J. Klaine (Steve) our favorite Ecotoxicology professor.
ACKNOWLEDGEMENTS

I am sincerely thankful to my advisor Dr. William S. Baldwin for guiding me throughout the whole PhD process. I would like to thank all my committee members Dr. Lisa J. Bain, Dr. Stephen J. Klaine, Dr. Gerald A. LeBlanc and Dr. Peter van den Hurk for providing me with feedback and help for the completion of the Doctoral program. I want to thank Dr. Patrick Gerard for providing me with the statistical analysis of my data of chapters 3, 4 and 5 of this thesis. My sincere thanks go out to Kansas Lipidomics Research Center and Mount Desert Island Biological Laboratory (Maine) for collaborations in this project.

I want to thank my past and current lab-members: Gautam Kumar Ginjupalli, Elizabeth Litoff, Delaney Reardon, Misha Sharif, Allie Cooper, Ramiya Kumar, Tyler Boswell, Charles Mansfield and Allison Schmidt. Gautam has been such a great teacher, Ramiya has been an amazing friend and Allison has been a huge support my last year at grad school. I would like to acknowledge my colleagues and friends from Rice-lab, Bain-lab and Klaine-lab. Special thanks to some of my friends in the department for their constant support through grad school: Ray Jui Tung Liu, Amy Anderson, Maria Rodgers and Sarah Au. I am very thankful for all the classes I have taken and mentoring I have received from different faculty members from Environmental Toxicology, Microbiology, Biological Sciences, EEES and CAFLS.

I would like to thank my funding source National Institute of Environmental Health Studies (NIEHS): ES017321. I am also very thankful for the SETAC Training Exchange Opportunity Award I received from SETAC (North America) to pursue
summer research at MDIBL (Maine). I would like to thank the Department of Biological Sciences for the confirmed Teaching Assistantship funding I received during my term in graduate school, and special thanks to Chris Minor for letting me teach Biology 1200 lab. I am very grateful for the travel awards I have received from Clemson University Graduate Student Government, Carolina SETAC and North America SETAC to present my research at various conferences during these past five years.

I want to thank my amazing friends from the Clemson University family and I would specifically like to mention my best friends Chirag Ojha and Nikeetha Dsouza for their beautiful friendship and guidance. I would like to say a big thank you to my fiancé Nick Watts for being my constant inspiration and support. Most important of all, my parents Mrs. Namita Sengupta and Mr. Ranen Sengupta – I couldn’t have done this without their love, trust and prayers. Finally a big thank you to my sister and mentor Ronita Sengupta who made me believe in my dreams. Thank you all.
<table>
<thead>
<tr>
<th>TABLE OF CONTENTS</th>
</tr>
</thead>
<tbody>
<tr>
<td>TITLE PAGE .................................................................................................................... i</td>
</tr>
<tr>
<td>ABSTRACT ..................................................................................................................... ii</td>
</tr>
<tr>
<td>DEDICATION ................................................................................................................ iv</td>
</tr>
<tr>
<td>ACKNOWLEDGEMENTS ..................................................................................................... v</td>
</tr>
<tr>
<td>LIST OF FIGURES ......................................................................................................... x</td>
</tr>
<tr>
<td>LIST OF TABLES ........................................................................................................... xii</td>
</tr>
</tbody>
</table>

**CHAPTER**

**I. INTRODUCTION** ........................................................................................................ 1

*Daphnia* as an ecotoxicology model ................................................................. 1
HR96 .................................................................................................................... 3
HR96 modulators ................................................................................................. 6
Aquatic organisms and stressors .......................................................................... 8
Diet Restriction .................................................................................................. 10
Polyunsaturated fatty acids (PUFAs) ................................................................. 11
Specific Aims..................................................................................................... 12

References............................................................................................................. 19

**II. THE HR96 ACTIVATOR, ATRAZINE, REDUCES SENSITIVITY OF *D. MAGNA* TO TRICLOSAN AND DHA** .............................................................................. 27

Abstract ....................................................................................................................... 27

Introduction .................................................................................................................... 28

Materials & Methods ..................................................................................................... 31

*Daphnia* culture ................................................................................................. 31
RNA extraction and qPCR ................................................................................. 31
Acute toxicity assays .......................................................................................... 32
Predictive mixture modeling .............................................................................. 33

[^14C]Testosterone glucosyltransferase assay ..................................................... 33
Anti-oxidant and reactive oxygen species assays .............................................. 34

Results............................................................................................................................ 35

HR96 expression ................................................................................................... 35
Single chemical toxicity tests .............................................................................. 36
Mixtures with LA mimic independent joint action ............................................... 37
Atrazine provides protection from HR96 inhibitors ........................................... 39
Atrazine induces anti-oxidant protection ........................................................... 41
TABLE OF CONTENTS (Continued)

Discussion ...................................................................................................................... 44
References ...................................................................................................................... 47

III. PERTURBATIONS IN POLAR LIPIDS, STARVATION SURVIVAL AND REPRODUCTION FOLLOWING EXPOSURE TO UNSATURATED FATTY ACIDS OR ENVIRONMENTAL TOXICANTS IN DAPHNIA MAGNA .............................. 54
Abstract .......................................................................................................................... 54
Introduction .................................................................................................................... 55
Materials and Methods ................................................................................................... 59
  *Daphnia magna* culture .............................................................................................. 59
  Chemicals ...................................................................................................................... 60
  Chronic toxicity tests .................................................................................................. 60
  Starvation Assay ........................................................................................................ 61
  Cholesterol analysis .................................................................................................... 62
  Lipid extraction and lipidomic analysis ....................................................................... 63
Results ............................................................................................................................ 64
  Reproductive toxicity during 21-day chemical exposures ............................................. 64
  Chemical-induced changes in starvation survival and fecundity in *D. magna* ....... 65
  Chemical exposures alter cholesterol levels and polar phospholipids .................... 68
Discussion ...................................................................................................................... 76
References ...................................................................................................................... 83

IV. EXCHANGE OF POLAR LIPIDS FROM ADULTS TO NEONATES IN DAPHNIA MAGNA: PERTURBATIONS IN LIPID ALLOCATION BY DIETARY LIPIDS AND ENVIRONMENTAL TOXICANTS ............................................................................. 91
Abstract .......................................................................................................................... 91
Introduction .................................................................................................................... 92
Materials and Methods ................................................................................................... 95
  *Daphnia magna* culture .............................................................................................. 95
  Chemicals ...................................................................................................................... 95
  Starvation survival - Reproduction assay ................................................................. 96
  Cholesterol and triacylglyceride (TAG) analysis ......................................................... 97
  Lipid extraction and lipidomic analysis of adult and neonatal daphnids ................. 98
  RNA extraction and qPCR ....................................................................................... 99
  Acute and chronic toxicity of carmofur and GW4869 .............................................. 100
Results .......................................................................................................................... 102
  Neonates preferentially sequester sphingomyelin .................................................... 102
  Starvation survival and fecundity in adult *D. magna* ............................................. 103
  Cholesterol and TAG levels in adult daphnids pre-exposed to chemicals .............. 107
  Fatty acids and toxicants decrease phosphatidylcholine and increase sphingomyelin levels in daphnids ................................................................. 109
TABLE OF CONTENTS (Continued)  

<table>
<thead>
<tr>
<th>Topic</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxicant and lipid-induced changes in sphingomyelin concentrations in adults and neonates</td>
<td>117</td>
</tr>
<tr>
<td>Perturbations in the regulation of genes involved in lipid absorption and sphingomyelin metabolism</td>
<td>119</td>
</tr>
<tr>
<td>Ceramidase inhibitor Carmofur alters fecundity in D. magna</td>
<td>124</td>
</tr>
<tr>
<td>Discussion</td>
<td>126</td>
</tr>
<tr>
<td>References</td>
<td>132</td>
</tr>
</tbody>
</table>

V. LOBSTERS ARE NOT SENSITIVE TO JUVENILE HORMONE ANALOGS.... 138

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>138</td>
</tr>
<tr>
<td>Introduction</td>
<td>138</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>140</td>
</tr>
<tr>
<td>Results</td>
<td>143</td>
</tr>
<tr>
<td>Discussion</td>
<td>147</td>
</tr>
<tr>
<td>References</td>
<td>149</td>
</tr>
</tbody>
</table>

VI. DISCUSSION ........................................................................................................ 153

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>References</td>
<td>160</td>
</tr>
</tbody>
</table>

APPENDICES ............................................................................................................. 162

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Supplementary figures from Chapter Two</td>
<td>163</td>
</tr>
<tr>
<td>B. Supplementary figures from Chapter Three</td>
<td>164</td>
</tr>
<tr>
<td>C. Supplementary figures from Chapter Four</td>
<td>174</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1 Acute toxicity and dose-response curves of select xenobiotic and fatty acid HR96 modulators to <em>D. magna</em></td>
<td>36</td>
</tr>
<tr>
<td>2.2 The toxicity of Linoleic acid (LA) is accurately predicted in a two-chemical mixture containing an HR96 inhibitor (triclosan and DHA)</td>
<td>38</td>
</tr>
<tr>
<td>2.3 The HR96 activator, atrazine induces a concentration-dependent interaction that protects daphnids from toxicity by triclosan or DHA</td>
<td>40</td>
</tr>
<tr>
<td>2.4 Testosterone glucosylation activity in atrazine exposed daphnids</td>
<td>41</td>
</tr>
<tr>
<td>2.5 Measurement of GST activity, antioxidant capacity and ROS (TBARS) induced by chemicals and chemical mixtures</td>
<td>43</td>
</tr>
<tr>
<td>3.1 Outline of the experimental design and timeline for the treatments and starvation of daphnids as described in the Materials and Methods</td>
<td>62</td>
</tr>
<tr>
<td>3.2 Starvation survival in <em>D. magna</em> following exposure to DHA, LA, atrazine or triclosan</td>
<td>66</td>
</tr>
<tr>
<td>3.3 Cholesterol concentrations and relative polar lipid levels in daphnids following four days of exposure to different chemicals</td>
<td>71</td>
</tr>
<tr>
<td>3.4 Changes in phosphatidylethanolamine (PE) or phosphatidylcholine (PC) composition among different exposure groups</td>
<td>73</td>
</tr>
<tr>
<td>3.5 Principal component analysis reveals associations between chemical exposures and lipid profiles in <em>D. magna</em></td>
<td>76</td>
</tr>
<tr>
<td>4.1 Timeline for chemical exposures, starvation and polar lipid determinations in <em>D. magna</em> as described in Materials and Methods</td>
<td>97</td>
</tr>
<tr>
<td>4.2 Relative concentrations of different classes of polar lipids in untreated adult and neonatal daphnids</td>
<td>103</td>
</tr>
<tr>
<td>4.3 Adult starvation survival, reproduction and neonate starvation survival in daphnids exposed to DHA, LA, PA, atrazine or triclosan</td>
<td>106</td>
</tr>
<tr>
<td>4.4 Protein, cholesterol and TAG concentrations in seven-day old <em>D. magna</em> following four days of exposure to different chemicals</td>
<td>108</td>
</tr>
</tbody>
</table>
## LIST OF FIGURES (Continued)

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>Relative levels of different polar lipid classes in adult and neonatal daphnids following four days of chemical exposures</td>
<td>113</td>
</tr>
<tr>
<td>4.6</td>
<td>Significant changes in relative polar lipid species among neonatal daphnids</td>
<td>115</td>
</tr>
<tr>
<td>4.7</td>
<td>Changes in sphingomyelin (SM) composition among different exposure groups in adult and neonatal daphnids</td>
<td>118</td>
</tr>
<tr>
<td>4.8</td>
<td>Altered expression of HR96, magro, mannosidase, NPC1b and Cer2 in 8 day-old <em>D. magna</em> exposed to fatty acids or toxicants for 24 hours</td>
<td>121</td>
</tr>
<tr>
<td>4.9</td>
<td>Altered expression of HR96, magro, mannosidase, NPC1b and Cer2 in 11 day-old <em>D. magna</em> exposed to fatty acids or toxicants for 96 hours</td>
<td>123</td>
</tr>
<tr>
<td>4.10</td>
<td>Altered fecundity of adult daphnids exposed to carmofur or GW4869</td>
<td>125</td>
</tr>
<tr>
<td>4.11</td>
<td>Sphingomyelin metabolism pathway</td>
<td>127</td>
</tr>
<tr>
<td>5.1</td>
<td>Lobsters were cultured in natural Maine seawater in 1 pint mason jars under constant aeration as shown</td>
<td>141</td>
</tr>
<tr>
<td>5.2</td>
<td>Survival and development of lobsters from stage 1 to 4 following exposures to DHA, AA, DHA + pyriproxyfen (PF) and AA + PF</td>
<td>144</td>
</tr>
<tr>
<td>5.3</td>
<td>Survival of larval lobsters following exposure to pyriproxyfen, fenoxycarb and methoprene</td>
<td>146</td>
</tr>
<tr>
<td>6.1</td>
<td>Adverse Outcome Pathways of atrazine and triclosan summarizing results and effects observed in this research</td>
<td>160</td>
</tr>
<tr>
<td>Table</td>
<td>Page</td>
<td></td>
</tr>
<tr>
<td>-------</td>
<td>------</td>
<td></td>
</tr>
<tr>
<td>1.1 HR96 inhibitors and activators among toxicants and unsaturated fatty acids</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>3.1 Survival of daphnids following release of broods</td>
<td>67</td>
<td></td>
</tr>
<tr>
<td>3.2 Quantity of each lipid type found in the polar lipids extracted from treated and untreated <em>D. magna</em></td>
<td>70</td>
<td></td>
</tr>
<tr>
<td>4.1 Quantity of each lipid group found in polar lipids extracted from treated and untreated adult <em>D. magna</em></td>
<td>110</td>
<td></td>
</tr>
<tr>
<td>4.2 Quantity of each lipid type found in the polar lipids extracted from treated and untreated neonatal <em>D. magna</em> (per brood)</td>
<td>111</td>
<td></td>
</tr>
<tr>
<td>4.3 Age-dependent expression of genes in untreated <em>D. magna</em></td>
<td>119</td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER ONE
INTRODUCTION

The purpose of this research is aimed at evaluating the effects of toxicants and diet restriction on toxicant sensitivity, development and reproduction in *Daphnia magna*. Given that HR96 is both a sensor for toxicants and unsaturated fatty acids, we concentrated our efforts on chemicals that activate or block HR96 (Karimullina et al., 2012). HR96 is an ortholog of constitutive androstane receptor (CAR), pregnane-X-receptor (PXR) or vitamin-D-receptor (VDR) that is found higher organisms, and as such is also a xenosensor (King-Jones et al., 2006) and energy sensor (Sieber and Thummel, 2009, 2012) similar to CAR and PXR (Ueda et al., 2002; Maglich et al., 2004; Nakamura et al., 2007). Chemicals used in this research are anthropogenic and dietary (fatty acid), HR96 inhibitors (triclosan, docosahexaenoic acid [DHA]) and activators (linoleic acid [LA], atrazine) (Karimullina et al., 2012). Therefore, we can examine the interplay between diet and chemical exposures. In addition, we investigated how toxicants or specific fatty acids may alter the allocation of lipid resources using reproduction, starvation survival, lipidomics, and gene expression as endpoints.

1.1 *Daphnia* as an ecotoxicology model

*Daphnia* are small planktonic crustaceans, widely used for toxicology testing (LeBlanc, 1984). *Daphnia* is a very important species to demonstrate adaptation to environmental stresses, spanning from the individual to the population level (Fernández-
Daphnia becomes an obvious choice for toxicology research because it is easy to culture in the laboratory (Adema, 1978). In order to understand the effects of chemical exposures in the aquatic ecosystem Daphnia is a preferred test organism because of its low to medium placement in the food chain and it is a major food source for fish (Lazorchak et al., 2009). Tests conducted by various toxicology groups with Daphnia of different species have shown that the results generated are comparable and daphnids are usually more sensitive than fish to toxicants (American Waterworks Association, 1975; Lazorchak et al., 2009). Because Daphnia undergoes clonal reproduction they are a great tool for quantitative genetic studies and help in the understanding of evolutionary ecology (Ebert, 2005). The two most popularly used species in aquatic toxicity testing and ecological studies are Daphnia pulex and Daphnia magna (USEPA, 2002). D. magna reproduces by cyclical parthenogenesis where males can contribute towards the genetic makeup of the neonates born during sexual stage of reproduction whereas D. pulex may opt for cyclic or obligate parthenogenesis where zygotes undergo ameiotic parthenogenesis inside the ephippium leading to no genetic contribution from the males (Rand et al., 1995).

Daphnia pulex is the first crustacean whose genome has been completely sequenced, with 200 megabases and 30,907 genes (Colbourne et al., 2011). The Daphnia magna genome is currently being sequenced, link to which has been provided here: http://server7.wfleabase.org:8091/gbrowse/cgi-bin/gbrowse/daphnia magna2/. D. pulex and D. magna possess mostly the same nuclear receptors with some NRs such as HR96
and retinoid X receptor (RXR) showing strong conservation among the two (Litoff et al., 2014).

*Daphnia* genome is being extensively studied in order to understand the interactions between ecologically responsive genes and the environment (Shaw et al., 2008). Thus sequencing the genome is a huge contribution in the realm of ecology, evolution, and environmental toxicology research (Schaack, 2008). The discovery of genes involved in xenobiotic metabolism in this species will help in understanding how this organism adapts to environmental toxicants and other stressors (Shaw et al., 2007). *Daphnia pulex* has 75 cytochrome P450 genes, many of which are involved in toxicant metabolism (Baldwin et al., 2009), and 26 nuclear receptors (Litoff et al., 2014) that regulate transcriptional responses to endogenous and exogenous ligands. Furthermore, 64 ATP binding cassette transporters have been discovered in *Daphnia pulex* that help in providing biochemical defense against chemicals by transporting them from cells (Sturm et al., 2009). These genes provide the basic mechanisms for response to internal and external stressors some of which may be dietary and others anthropogenic.

### 1.2 HR96

Nuclear Receptors are ligand activated transcription factors (Kretschmer and Baldwin, 2005; Hernandez et al., 2009). Nuclear receptors recognize specific ligands (i.e. steroid hormones, toxicants, bile acids, fatty acids) and in turn transcriptionally alter protein expression (Parks et al., 1999; Staudinger et al., 2001; Moreau et al., 2008). PXR
and CAR are involved in regulation of phase I to III metabolic enzymes responsible for the clearance of xenobiotics and steroids (Ueda et al., 2002) (Rosenfeld et al., 2003). Research has also demonstrated the involvement of both these receptors in energy metabolism and immune response pathways, henceforth having several clinical implications (Hernandez et al., 2009; Gao and Xie, 2012). CAR activation by phenobarbital down-regulates fatty acid oxidation and glucose synthesis (Ueda et al., 2002). Activation of PXR by pregnenolone 16 alpha-carbonitrile (PCN) downregulates beta-oxidation and ketogenesis also increasing lipogenesis (Nakamura et al., 2007). There is also a cross-talk between CAR/PXR and insulin or glucagon responsive transcription factors such as forkhead box O1, forkhead box A2 and cAMP-response element binding protein (Konno et al., 2008; Gao and Xie, 2012).

HR96 is an ortholog of CAR and PXR that controls the expression of metabolic and stress response genes in invertebrates (King-Jones et al., 2006). In Drosophila, HR96 activation has been shown to induce detoxification genes important in the metabolism of several toxicants (King-Jones et al., 2006; Lin et al., 2011). Drosophila HR96 modulates phenobarbital induction of Cyp6d1; and Drosophila which lacks HR96 does not induce detoxification genes in response to phenobarbital (King-Jones et al., 2006). HR96 activation induces Cyp6d1 (Lin et al., 2011), a key detoxification gene known to be involved in resistance to pyrethroids (Liu and Scott, 1998). It is a promiscuous nuclear receptor that can be activated by some n-6 and n-9 (omega-6 and omega-9) unsaturated fatty acids and several toxicants (Karimullina et al., 2012). HR96 is also inhibited by several chemicals including triclosan and some n-3 (omega-3) unsaturated fatty acids.
HR96 plays an important role in cholesterol and triacylglycerol (TAG) homeostasis in *D. melanogaster* (Sieber and Thummel, 2009, 2012). Overexpression of HR96 leads to resistance from starvation and an increase in TAG concentrations, and this is regulated through gastric lipase (Magro; CG5932). The TAG lipase activity of Magro is present in the anterior end of the gut and it helps in the uptake of dietary fats in the intestinal lumen (Sieber and Thummel, 2009). Magro is also required in the intestine of *Drosophila* to maintain cholesterol homeostasis as it increases clearance of cholesterol (Bujold et al., 2010).

HR96 is also a key regulator of the Niemann Pick type C gene family involved in cholesterol and fatty acid homeostasis (especially sphingolipids), as many Niemann Pick genes are sphingomyelinases or sphingomyelin carriers (Horner et al., 2009; Jenkins et al., 2009). Niemann Pick disease is a lysosomal storage disorder caused by the improper retention of sphingomyelin (Callahan and Khalil, 1976). The metabolism of sphingomyelins is important in cell signaling and development (Kitatani et al., 2008). For example, the central metabolite of sphingomyelin metabolism is ceramide which plays a crucial role in cell growth, differentiation and also senescence (Venable et al., 1995). Therefore, perturbation of HR96 activity may have sublethal effects on resource allocation or effects on maturation and reproduction; some of which may only be manifested during specific environmental or dietary conditions when there is competition between dietary and anthropogenic activators of HR96.
1.3. HR96 modulators

Among the several chemicals tested for HR96 activation or inhibition (Karimullina et al., 2012) some have been listed below (Table 1.1). The chemicals showed a specific trend in their role as HR96 modulators, with pesticides being HR96 activators and pharmaceuticals being HR96 inhibitors although the sample size is small. Interestingly, fatty acids were also grouped under HR96 inhibitors or activators. The n-3 unsaturated fatty acids are HR96 inhibitors and the n-6 and n-9 unsaturated fatty acids are HR96 activators (Table 1.1).
Table 1.1: HR96 activators and inhibitors among toxicants and unsaturated fatty acids (Karimullina et al. 2012)

<table>
<thead>
<tr>
<th>Chemical Modulators of HR96 Nuclear Receptor</th>
<th>Effect on DappuHR96</th>
<th>EC 50 (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Corticosterone</td>
<td>Activates</td>
<td></td>
</tr>
<tr>
<td>Androstanol</td>
<td>Inhibits</td>
<td></td>
</tr>
<tr>
<td>Estradiol</td>
<td>Activates</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>Activates 1.84</td>
<td></td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>Activates</td>
<td></td>
</tr>
<tr>
<td>Pyriproxyfen (JHA)</td>
<td>Activates</td>
<td></td>
</tr>
<tr>
<td>M-Methyl Arsonate</td>
<td>Activates</td>
<td></td>
</tr>
<tr>
<td>Phenobarbital</td>
<td>Inhibits</td>
<td></td>
</tr>
<tr>
<td>Fluoxetine</td>
<td>Inhibits</td>
<td></td>
</tr>
<tr>
<td>Triclosan</td>
<td>Inhibits 12</td>
<td></td>
</tr>
<tr>
<td>Corn Oil (n-6)</td>
<td>Activates</td>
<td></td>
</tr>
<tr>
<td>Linoleic Acid (n-6)(LA)</td>
<td>Activates</td>
<td></td>
</tr>
<tr>
<td>Oleic Acid (n-9)</td>
<td>Activates</td>
<td></td>
</tr>
<tr>
<td>Alpha-linolenic Acid (n-3) (ALA)</td>
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<td>Eicosapentaenoic Acid (n-3)(EPA)</td>
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<td>Docosahexaenoic acid (n-3)(DHA)</td>
<td>Inhibits 35.6</td>
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The HR96 modulators used in our research are docosahexaenoic acid (DHA), linoleic acid (LA), atrazine and triclosan. DHA is n-3 fatty acid and HR96 inverse agonist in *Daphnia* (Karimullina et al., 2012) and a CAR inverse agonist in rats (Li et al., 2007),
as it represses constitutive HR96 and CAR action. LA is a weak to moderate activator of HR96 in *Daphnia* (Karimullina et al., 2012) and CAR in mice (Finn et al., 2009) that can be converted to AA (Nakamura and Nara, 2004). Among the toxicants in our research triclosan, a widely used antimicrobial agent in consumer products such as toothpaste, hand-soap, acne-cream (Daughton and Ternes, 1999), and an HR96 inhibitor in *Daphnia* (Karimullina et al., 2012). The compound is of concern because it is often detected in wastewater (McAvoy et al., 2002) and sometimes in surface water (Kolpin et al., 2002). Atrazine is a triazine herbicide (Hayes et al., 2002) and is an HR96 activator (Karimullina et al., 2012). Atrazine is known to disrupt the endocrine system in vertebrates (Cooper et al., 2000) and may interact with other chemicals to induce mixture-toxicity (Phyu et al., 2011; Pérez et al., 2013; Chen et al., 2015; Yang et al., 2015). It has been proposed that atrazine increases dopamine and decreases norepinephrine levels in the hypothalamus; leading to reduction of luteinizing hormone and prolactin levels and increasing conversion of testosterone into 17β-estradiol (Stoker et al., 1999; Cooper et al., 2000).

1.4 Aquatic organisms and stressors

Aquatic organisms are exposed to multiple stressors at the same point of time (Scherer et al., 2013). These stressors could range from temperature variations, alterations in duration of day-night cycles, over-crowding, algal blooms, chemical spills or limited food resources (Casterlin and Reynolds, 1977; Bergman Filho et al., 2011; Glaholt et al., 2012; Masclaux et al., 2012). Organisms may have unique methods of dealing with these stressors and in turn allocate their energy resources towards certain
physiological responses (survival, growth, reproduction) depending on life stage, seasonal cues, or amount of food (Latta IV et al., 2011; Coutellec and Barata, 2013). Effects of several individual stressors have been investigated in different organisms. However scientists still find it challenging to predict and understand the effects of multiple stressors; recent research has looked into the potential of predictive mixture modelling approaches in toxicology (Olmstead and LeBlanc, 2005; Glaholt et al., 2012; Scherer et al., 2013). As toxicologists are often involved in understanding the mechanisms of action of anthropogenic compounds, other abiotic and biotic factors in the ecosystem may be neglected in the study. An organism may respond very differently to a chemical contaminant, based on its co-exposure to a biotic or abiotic stressor.

There is an increasing interest to understand how diet plays a role in determining toxicant sensitivity (Ginjupalli et al., 2015). In a natural ecosystem, organisms are exposed to different kinds of diets at different times of the year (Casterlin and Reynolds, 1977). One such example is algal succession in freshwater ecosystems as the species of algae change during the seasons so do the lipid and protein content of the food (Guedes et al., 2011; Hartwich et al., 2012). This may have an impact on primary and secondary consumers (Casterlin and Reynolds, 1977; Burkepile and Hay, 2010). This variation has the potential of acting as a biotic stressor as the diet may interact differently with key lipid receptors, including different or similar nuclear receptors (Chawla et al., 2001). For example, under dietary restriction changes in lipid composition may reduce the ability of an organism to respond to xenobiotic HR96 signaling and increase their sensitivity to the toxicant. It is important to understand interactions between biotic and abiotic stressors,
and *Daphnia* with its well understood ecology, provides an excellent model for linking ecology/physiology to specific metabolic and genomic changes (Colbourne et al., 2011).

### 1.5. Diet Restriction

Organisms may be exposed to limited dietary food sources and in response to that they may tend to allocate resources towards survival or reproduction. “Lifespan expansion” under diet restriction condition has been demonstrated in some species (Mair and Dillin, 2008). In certain species such as mice, *C. elegans* and *D. melanogaster* diet restriction can induce positive effects on lifespan expansion and may reduce fecundity and reproductive maturity (Mair and Dillin, 2008). Organisms such as *D.magna* can alter their life-history strategies based on available food resources, and therefore they become a suitable model for understanding these responses (Latta IV et al., 2011). For example, under dietary restriction *Daphnia* often produce fewer, but larger offspring while under ideal dietary conditions the *Daphnia* produce greater number of offspring but they are smaller and may be sensitive to less than ideal conditions (Tessier et al., 1983; Tessier and Consolatti, 1989). It is also speculated that diet plays a role in understanding how an organism may respond to certain toxicants (Archer et al., 2001). Past research has shown food level impacts toxicity due to insecticide imidacloprid and nickel in *D. magna* only under starvation conditions (Pavlaki et al., 2014). Past research has also demonstrated algae composition and lipid concentrations may impact pesticide-induced male production in *D. magna* (Ginjupalli et al., 2015).
1.6. Polyunsaturated fatty acids (PUFAs)

Lipids form an integral part of diet and are capable of impacting metabolic rate, neuropeptide activity, second messenger generation and gene expression (Ghosh et al., 1997; Hulbert et al., 2005). This is essentially because of the role of PUFAs as signaling molecules for growth, development and energy metabolism (Hulbert et al., 2005; Wei et al., 2010; Catala, 2013). In context of our research we are interested in understanding the role of PUFA in the health of aquatic organisms such as *D.magna*. Both invertebrates and vertebrates feed on phytoplankton, and if it is rich in PUFA it is associated with better growth and reproduction in these organisms (Verreth et al., 1994; Brett and Muller-Navarra, 1997). Specific PUFAs associated with growth and reproduction of aquatic organisms such as arthropods are arachidonic acid (AA; n-6 fatty acid), eicosapentaenoic acid (EPA, an n-3 fatty acid) and DHA (n-3 fatty acid) (Brett and Muller-Navarra, 1997; Ginjupalli et al., 2015). During food-shortage daphnids retain PUFAs such as EPA and AA (Brett et al., 2006). DHA is retained in the ovaries of *D. magna*, however some studies indicate DHA gets converted to EPA (Bunescu et al., 2010; Taipale et al., 2011).

Organisms can be exposed to both PUFAs (through diet) and toxicants (in the environment) at the same time, and they both may tend to compete for the same receptors (Zhai et al., 2010; Karimullina et al., 2012). In context of *D. magna* and HR96, toxicants could potentially interfere with the uptake of lipids and lead to perturbations in energy metabolism because these processes are in part regulated through the same receptors (Karimullina et al., 2012) and similar receptors may compete for other lipids or resources such as co-activators (Zhai et al., 2010; Li et al., 2014). Diet restriction or shortage can
also lead to altering an organism’s sensitivity to toxicants (Ginjupalli et al., 2015). For example, a poor diet increases pyriproxyfen-induced male production and AA protects from the male producing effects of pyriproxyfen under dietary restricted conditions (Ginjupalli et al., 2015).

1.7. Specific Aims

*Daphnia* are the first crustaceans to have their genome sequenced (Colbourne et al., 2011). This has increased our understanding about the molecular mechanisms by which *Daphnia* adapt to anthropogenic stressors and ecological changes. Nuclear receptors are ligand activated transcription factors and in turn respond to internal and external chemical cues (Chawla et al., 2001; Willson and Moore, 2002). HR96 is a toxicant receptor related to the vertebrate receptors, CAR and PXR, (Kretschmer and Baldwin, 2005). Similarly, HR96 is activated by several toxicants and some n-6 (omega-6) polyunsaturated fatty acids (Karimullina et al., 2012). HR96 is inhibited by some toxicants including triclosan and n-3 (omega-3) unsaturated fatty acids. HR96, CAR, and PXR induce the expression of numerous phase I – III detoxification proteins (King-Jones et al., 2006).

*Daphnia magna* like most organisms can respond to different ecological stressors (environmental contaminants and diet) through several different pathways. HR96 is involved in acclimation to xenobiotics (King-Jones et al., 2006), uptake and metabolism of cholesterol (Horner et al., 2009) and triacylglycerols (Sieber and Thummel, 2009). We hypothesized that diet and xenobiotics may interact to alter the sensitivity of *D.magna* to
individual or mixture of stressors, through induction of protective and developmental pathways; affecting the overall fitness of the organism because of changes in resource allocation that manifest themselves physiologically as changes in development, reproduction or survival.

Aim 1: Determine if HR96 inhibitors increase the toxicity of HR96 activators.

HR96 is activated and inhibited by different toxicants and dietary fatty acids (Karimullina et al., 2012). We hypothesized that HR96 inhibitors will block the protective responses of HR96 activators and in turn increase toxicity. We performed the following experiments under aim 1:

1. We performed forty-eight hour acute toxicity tests to determine LC50 values, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) associated with the following compounds: oleic acid, alpha-linolenic acid, cholesterol, DHA, LA, atrazine and triclosan.

2. We determined the age-dependent expression of HR96 at different age groups of daphnids (2, 4, 7 and 14 day old daphnids), to confirm which age group has the necessary HR96 to transcriptionally respond to the different chemical exposures which would be used for subsequent assays.

3. We compare forty-eight hour mixture toxicity tests with HR96 inhibitors (DHA or triclosan) and HR96 activators (LA or atrazine) with <24-hour old neonates, and compared percentage survival between the different mixture groups. We also predicted toxicity associated with each of the chemical mixtures using the
interactive hazard calculator, Computational Approach to the Toxicity Assessment of Mixtures (CATAM) developed by Dr. Gerald LeBlanc and his group at North Carolina State University, USA (in 2006).

4. In order to understand the metabolic and enzymatic pathways affected by these specific chemicals and their mixtures, we performed biochemical studies to complement our results following the in vivo acute toxicity assays. We evaluated the detoxification pathways altered by the individual chemicals or their mixtures.

Results from this part of our research have been published as


and is presented as chapter two in this thesis.

**Aim 2: Examine the effects of HR96 activators and inhibitors on starvation survival, reproduction, and allocation of polar lipids**

In addition to HR96 acting as a toxicant receptor (Karimullina et al., 2012) (King-Jones et al., 2006), it is also a cholesterol and triglyceride sensor that regulates cholesterol homeostasis and increases absorption of triacylglycerols (Sieber and Thummel, 2009). Therefore, activation or inhibition of HR96 may have sublethal effects; potentially hormetical but also subtle effects on developmental time, reproduction, or growth. Some of these sublethal effects could be further perturbed by changes to the diet that occur during toxicant exposure. Diet and xenobiotics may interact to alter the
sensitivity of *D.magna* to stressors, through induction of protective response pathways. This will affect the overall fitness of the organism because of changes in resource allocation that manifest themselves physiologically as changes in development, reproduction, or survival. As HR96 regulates metabolism and depuration of both toxicants and fats, we were interested to determine if toxicants that bind to HR96 capable of disrupting energy or lipid allocation.

We hypothesized that HR96 activators (atrazine, LA) may increase xenobiotic metabolism and lipid absorption leading to increased starvation-survival and HR96 inhibitors (triclosan, DHA) may decrease xenobiotic metabolism and alter lipid absorption leading to the allocation of resources towards the ovaries for reproduction and development (or they may have no effect at all). Our goal in aim 2 was to determine if HR96 modulators alter resource allocation in adolescent *D. magna*. The study would help us determine if the organism utilizes resources for better survival, reproduction or transfers resources to neonates. We performed the following set of experiments under aim 2:

1. We performed twenty-one day chronic toxicity tests with each of the HR96 modulators (DHA, LA, atrazine and triclosan) to test if these compounds are able to affect reproduction in *D. magna* and at what concentrations. These chronic toxicity tests helped us to determine which concentrations of the individual chemicals we could use for further experiments in aims 2 and 3.

2. Neonatal daphnids were either untreated or exposed to DHA, LA, atrazine or triclosan for four days and fed normal *Daphnia* diet. They were taken out of exposures on Day 5
and starved for next few days until they died. We recorded their survival and reproduction during this time.

3. With a similar experimental set-up as above, we extracted polar lipids from daphnids on Day 5. We measured cholesterol levels and polar lipid profiles and associated these changes with reproduction and development of adolescent daphnids.

Research findings from this have been published as

Sengupta, N., Gerard, P.D., Baldwin, W.S., 2016. Perturbations in polar lipids, starvation survival and reproduction following exposure to unsaturated fatty acids or environmental toxicants in Daphnia magna. Chemosphere. 144, 2302-2311

and is presented as chapter three in this thesis.

Aim 3: Determine the differences in lipid allocation between exposed neonates and adults. Here we found some unique differences in sphingomyelin metabolism consistent with changes in reproduction and development

HR96 is known to play a role in sphingolipid metabolism in Drosophila and because sphingolipids have been suggested to be associated with Niemann Pick disease it may be possible that HR96 plays a direct role in regulation of genes involved in the similar pathway (Bujold et al., 2010). Because aim 2 demonstrated that toxicants could alter lipid profiles and use of energy in adolescent daphnids by chemical specific means, we wanted to perform further experiments with adult and neonatal daphnids to understand developmental differences between neonates and adults that may provide
clues if these compounds alter lipid profiles in a similar fashion and if there is any exchange of specific lipids between adults and neonates. We also wanted to determine if specific chemicals which altered certain phenotypes and lipid signatures in our study could alter expression of genes which are regulated by HR96. We performed the following experiments under aim 3:

1. We exposed adult daphnids (seven-day old) to different chemical treatments (the ones used in aim 2 and also palmitic acid – a lipid control) and then starved them from Day 11 onwards until they died. During starvation we recorded data for survival and reproduction every day. On day 16 (after 5 days of starvation) we collected neonates born from these starved adults and followed them for survival.

2. On day 11 (with similar experimental set-up as above) we extracted samples from adult daphnids for lipidomic analysis, cholesterol and triacylglycerol measurements. Polar lipids were also extracted from neonates born from 11-day old adults. Cholesterol, triglycerides, and polar lipid profiles were determined to test whether different toxic chemicals or fatty acids altered specific lipid pathways.

3. We extracted RNA from 11-day old adults (exposed to different chemicals for 96 hours) and also 8-day old adults (exposed to different chemicals for 24 hours). We investigated changes in expression of biomarker genes of HR96 by different chemicals and fatty acids in adult daphnids, and tried to associate these changes with reproduction and development in *D. magna*. 
Research findings from this have been presented in chapter four of this thesis and the manuscript titled “Exchange of Polar Lipids from Adults to Neonates in Daphnia magna: Perturbations in Lipid Allocation by Dietary Lipids and Environmental Toxicants” will be submitted to Environmental Science and Technology soon. I will be first author.

**Aim 4: Investigate the benefits and toxicity of juvenile hormone analogs in the presence or absence of polyunsaturated fatty acids on decapod crustacean American lobster (Homarus americanus).**

It is essential to understand the implications of individual contaminants, contaminant mixtures, and dietary resources on the health of higher crustaceans (shrimps, crabs or lobsters), which form an essential component of the food chain and are commercially important species and human food sources. We were specifically interested in understanding how diet mitigates or enhances toxicity, and perturbs growth and development during single chemical and concurrent exposures to juvenile hormone analogs. We performed the following two experiments with larval lobsters to determine these effects:

1. We investigated the effects of individual juvenile hormone analogs (pyriproxyfen, methoprene and fenoxycarb) on survival and development of larval lobsters (stage 1 to 4).
2. We also determined the effects of JHAs on lobsters in the presence of polyunsaturated fatty acids (PUFAs) such as DHA and arachidonic acid (AA) which are known to play a role in crustacean development and reproduction.

This project was performed as a part of a short-term summer research project at Mount Desert Island Biological Laboratory, Bar Harbor, ME. Results from this part of the study have been presented as a part of chapter five in this thesis.

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CHAPTER TWO

THE HR96 ACTIVATOR, ATRAZINE, REDUCES SENSITIVITY OF D. MAGNA TO TRICLOSAN AND DHA

2.1 Abstract

HR96 is a CAR/PXR/VDR ortholog in invertebrates, and a promiscuous endo- and xenobiotic nuclear receptor involved in acclimation to toxicants. *Daphnia* HR96 is activated by chemicals such as atrazine and linoleic acid (LA) (n-6 fatty acid), and inhibited by triclosan and docosahexaenoic acid (DHA)(n-3 fatty acid). We hypothesized that inhibitors of HR96 may block the protective responses of HR96 based on previously performed luciferase assays. Therefore, we performed acute toxicity tests with two-chemical mixtures containing a HR96 inhibitor (DHA or triclosan) and a HR96 activator (LA or atrazine). Surprisingly, results demonstrate that triclosan and DHA are less toxic when co-treated with 20-80 µM atrazine. Atrazine provides concentration-dependent protection as lower concentrations have no effect and higher concentrations cause toxicity. LA, a weaker HR96 activator, did not provide protection from triclosan or DHA. Atrazine’s protective effects are presumably due to its ability to activate HR96 or other toxicologically relevant transcription factors and induce protective enzymes. Atrazine did not significantly induce glucosyltransferase, a crucial enzyme in triclosan detoxification. However, atrazine did increase antioxidant activities, crucial pathways in triclosan’s toxicity, as measured through GST activity and the TROLOX equivalence assay. The increase in antioxidant capacity is consistent with atrazine providing
protection from a wide range of toxicants that induce ROS, including triclosan and unsaturated fatty acids predisposed to lipid peroxidation.

2.2 Introduction

NRs are transcription factors that are often activated by a diverse set of ligands, including steroid hormones, bile acids, fatty acids, and toxicants (Hernandez et al., 2009). In turn, they transcriptionally regulate the expression of specific proteins. NRs such as the PXR and CAR are involved in the regulation of phase I to III metabolic enzymes responsible for the clearance of xenobiotics and steroids (Ueda et al., 2002; Rosenfeld et al., 2003). Recent research also demonstrates the involvement of both these receptors in energy metabolism and immune response pathways (Hernandez et al., 2009).

Daphnia are widely used for toxicology testing, and the sequencing of the Daphnia pulex genome has assisted in our understanding of how the environment and genomes interact to help organisms acclimate and adapt (Colbourne et al., 2011; De Coninck et al., 2014). Both Daphnia magna and D. pulex have 26 nuclear receptors, including a HR96 (Litoff et al., 2014). HR96 is an ortholog of CAR, PXR and VDR (Litoff et al., 2014), and controls the expression of metabolic and toxicant stress response genes in invertebrates, including CYPs, carboxylesterases, GSTs, and glucosyltransferases (King-Jones et al., 2006). HR96 also plays an important role in TAG homeostasis in Drosophila melanogaster, through its regulation of CG5932 gastric lipase (Magro). Magro is also required in the intestine of Drosophila to maintain cholesterol homeostasis by increasing its clearance (Sieber and Thummel, 2012). D. pulex HR96 is a
promiscuous nuclear receptor that is activated by several toxicants such as atrazine, chlorpyrifos and pyriproxyfen, and is also activated by several n-6 and n-9 (omega-6 and 9) unsaturated fatty acids (Karimullina et al., 2012). In addition, HR96 is inhibited by some chemicals including triclosan, phenobarbital and fluoxetine, and the n-3 (omega-3) unsaturated fatty acids, DHA and EPA (Karimullina et al., 2012).

The n-3 fatty acid, DHA is an efficacious HR96 inverse agonist in Daphnia (Karimullina et al., 2012) and a CAR inverse agonist in rats (Li et al., 2007), as it represses constitutive HR96 and CAR action. LA is a weak to moderate activator of HR96 in Daphnia (Karimullina et al., 2012) and CAR in mice (Finn et al., 2009). Triclosan is a widely used antimicrobial agent found in consumer products, including soap, deodorant, toothpaste, mouthwash and shampoo (Daughton and Ternes, 1999). It has been detected both in wastewater (McAvoy et al., 2002) and surface water (Kolpin et al., 2002). Atrazine is a triazine herbicide that can affect endocrine signaling pathway in vertebrates (Cooper et al., 2000). It has been reported to cause demasculinization of male frogs (Xenopus laevis) (Hayes et al., 2002) and structural disruption in testis of male goldfish (Carassius auratus) (Spanò et al., 2004). Atrazine affects endocrine signaling by increasing dopamine and reducing norepinephrine concentrations in the hypothalamus, and in turn reducing luteinizing hormone and prolactin levels. This leads to increased conversion of testosterone into 17β-estradiol (Stoker et al., 1999; Cooper et al., 2000).

Activation of CAR and PXR induces a number of detoxification enzymes (Hernandez et al., 2009). This positive regulation can be protective (Mota et al., 2010); however, CAR and PXR are both associated with drug-drug interactions because of the
induction of key enzymes. For example, CAR activation increases acetaminophen toxicity (Zhang et al., 2002). PXR activation is associated with increased clearance of warfarin (Mu et al., 2006) ethinyl estradiol, (Hall et al., 2003), and immunosuppressants (Hauser et al., 2012) leading to clotting, unintended pregnancies, and rejection of organ transplants. Because HR96 is also promiscuous (Karimullina et al., 2012) and involved in the regulation of similar pathways and enzymes (King-Jones et al., 2006), we predicted that chemicals that alter HR96 activity may cause similar drug-drug or toxicant-toxicant interactions.

Previously performed transactivation assays demonstrated that triclosan and DHA repress the activation of HR96 in a dose-dependent manner (Karimullina et al., 2012), and in turn, several HR96 activators such as atrazine, LA, pyriproxyfen and estradiol either did not increase luciferase activity in their presence or their activity was significantly repressed. Because of the widespread use of atrazine and triclosan and their pervasive presence in water bodies (Gilliom et al., 2006; Halden, 2014), they were used in this study to examine the potential opposing effects of HR96 modulators on the toxicant sensing and acclimation process in *Daphnia*. DHA and LA were examined as HR96 also responds to dietary unsaturated fatty acids (Karimullina et al., 2012). Therefore we tested the role these fatty acid exposures may have on toxicant responses; mitigating or enhancing toxicity in *D. magna*. We hypothesized that HR96 inhibitors will block the protective responses of HR96 activators and in turn increase toxicity.
2.3 Materials and Methods

2.3.1 Daphnia culture

*D. magna* were cultured in moderately hard water with a 16:8 light: dark cycle and a temperature between 21-23°C. The daphnids were fed the unicellular green algae, *Pseudokirchneriella subcapitata* (purchased from Aquatic Biosystems, Fort Collins, CO and cultured in the laboratory), and supplemented with TetraFin (Masterpet Corp., New South Wales, Australia) (Ginjupalli and Baldwin, 2013).

2.3.2 RNA extraction and qPCR

Four different age groups (2, 4, 7 and 14-day old) of *D. magna* were euthanized, RNA extraction was performed using an RNAeasy mini kit (Qiagen, Germantown, MD), and RNA quantified with a spectrophotometer at 260/280 nm. cDNA was synthesized from the RNA samples (2 µg RNA) with MMLV reverse transcriptase. qPCR was performed according to MIQE standards (Bustin et al., 2009) to determine the expression of HR96 in the daphnids with forward primer 5’-TCT-GCG-ACA-AGG-CTT-TAG-GTT-3’ and reverse primer 5’-AGG-GCA-TTC-CGT-CTA-AAG-AAG-GCT-3’ at an annealing temperature of 58°C. β-actin was used as the housekeeping gene with forward primer 5’-CCA-CAC-TGT-CCC-CAT-TTA-TGA-AG-3’ and reverse primer 5’-CGC-GAC-CAG-CCA-AAT-CC-3’ at an annealing temperature of 52.2°C as described previously (Heckmann et al., 2006). Efficiencies of the reactions were determined based on standard curves from 1:1, 1:5, 1:25, 1:125, 1:625 and 1:3125 dilutions of cDNA mixtures taken from all samples. The efficiency of the HR96 qPCR reaction varied
between 85 – 92%, and the efficiency of the β-actin qPCR varied between 88 - 102%. Samples were diluted 1:3 and quantified with 0.25X SYBR Green (Qiagen, Germantown, MD USA) using the iCycler from Bio-Rad Laboratories (Hercules, CA USA) (Mota et al., 2010). The results were normalized to the expression of β-actin. Gene expression was quantified by taking the efficiency curve of the reaction to the power of the threshold cycle (Ct) over the β-actin.

2.3.3 Acute toxicity assays

Forty-eight hour acute toxicity tests were conducted with LA (≥ 99 %), oleic acid (≥ 99%), α-linolenic acid (ALA) (≥ 99%), cholesterol (≥ 99%), DHA (≥ 98 %), triclosan (97%), and atrazine (98.9%) (Sigma-Aldrich, St. Louis, MO USA). Stock solutions of these chemicals were made in absolute ethanol (Sigma-Aldrich Chemical Co., Inc, Milwaukee, WI USA), except atrazine, which was dissolved in 99.7% dimethyl sulfoxide (Fisher Scientific, Fair Lawn, NJ USA). Toxicity was determined with individual chemicals and with two-chemical mixtures, with solutions made in moderately hard water. A variety of concentration ranges of individual chemicals were chosen to perform the first set of acute toxicity assays, in order to determine EC50 values using established protocols (USEPA, 2002) with four < 24-hour old daphnids in each 50 ml beaker and five beakers per treatment group. The EC50 values and 95% confidence interval were determined from the sigmoidal dose response curves, which were generated using GraphPad Prizm 4.0. In addition, the toxicity of chemical mixtures was determined with one HR96 activator and one HR96 inhibitor provided at different concentrations to the
daphnids. LA (2, 5 and 10 µM ) and atrazine (5, 10, 20, 40 and 80 µM ) were used as HR96 activators and DHA (2, 5 and 8 µM ) and triclosan (0.75, 1, 1.3 and 1.5 µM ) were used as HR96 inhibitors in the mixture assays. In these assays four < 24-hour old daphnids were used in each 50 ml beaker with ten beakers per treatment group.

2.3.4 Predictive mixture modeling

The interactive hazard calculator, Computational Approach to Toxicity Assessment of Mixtures (CATAM) (http://www.ncsu.edu/project/toxresearch/model5/) (Rider and LeBlanc, 2005) was used to predict toxicity associated with the chemical mixtures using the independent join action model with experimentally determined EC50 values (Olmstead and LeBlanc, 2005). The independent joint action model was used because the mechanism of action of the individual chemicals in the mixtures is not known and toxicity caused by each chemical probably does not occur through the same mechanism. HR96 inhibitors were assigned to cassette 1 of the model and HR96 activators were assigned to cassette 2 of the model. Toxicity of the chemical mixtures as measured during toxicity tests were compared to toxicity curves determined by the CATAM model.

2.3.5 [14C]Testosterone glucosyltransferase assay

Less than 48-hour old neonates (n = 5 beakers per treatment group; each beaker with ten daphnids) were pre-exposed to different concentrations of atrazine (0, 10, 20, 40 µM ) for 16 hours. Daphnids were exposed to the [14C]testosterone (2 mCi/mmol,
150,000 dpm/assay) for six hours. Ethyl acetate was used to extract testosterone and its lipophilic metabolites from the aqueous solution. Water fractions were collected from each tube, redissolved in 0.1 M NaAcetate, and conjugated testosterone was hydrolyzed with ten units of β-glucosidase for 2 h. Freed testosterone was extracted with ethyl acetate and the formerly glucosylated products were measured via liquid scintillation spectrophotometry and quantified according to previously published protocols (Baldwin et al., 1997).

2.3.6 Anti-oxidant and reactive oxygen species assays

Less than 48-hour old neonates (n = 4 beakers with 20 daphnids each) were exposed to individual chemicals or mixtures of chemicals (40 μM atrazine, 0.75 μM triclosan, 8 μM DHA, 40 μM atrazine + 0.75 μM triclosan and 40 μM atrazine + 8 μM DHA) for 16 hours. Daphnids were homogenized, and the supernatant collected and stored at -80°C for biochemical assays. Lipid peroxidation caused by ROS induced by chemical exposure was determined by measuring Thiobarbituric Acid Reactive Substances (TBARS) (Cayman Chemical Co., Ann Arbor, MI). Protein (10 μl) was incubated with thiobarbituric acid for 1 hour at 100°C. The reaction was stopped on ice for 10 minutes followed by centrifugation, and a 30 min incubation at room temperature before samples were read at fluorometrically at an excitation wavelength of 530 nm and an emission wavelength of 550 nm (Armstrong and Browne, 1994). Concentrations of malondialdehyde (μM) in the samples determined based on a prepared standard curve. Glutathione S-transferase (GST) activity was determined by incubating 5 μl of protein
with glutathione and 1-chloro 2, 4-dinitrobenzene (CDNB) for 10 min at room temperature. The formation of a dinitrobenzene-glutathione conjugate was measured at 340 nm (Baldwin and LeBlanc, 1996). The Trolox assay (Cayman Chemical Co., Ann Arbor, MI), which measures a number of anti-oxidant mechanisms was performed to measure the overall ability of *D. magna* to respond to ROS following exposure to individual chemicals or a two-chemical mixture. The anti-oxidant assay is based on the ability of the samples to inhibit metmyoglobin oxidation of 2,2’-azino-d-[3-ethylbenzthiazoline sulphonate] (ABTS) to ABTS$^+$ (Miller and Rice-Evans, 1997).

Briefly, 10 µl of protein sample was added to 10 µl of metmyoglobin, and 150 µl of chromagen. Incubations were initiated with 40 µl of hydrogen peroxide and ABTS$^+$ was measured after a 5 min incubation at room temperature at 750 nm. The antioxidant capacity of the samples was compared to that of a Trolox (a tocopherol analog) standard and the results were quantified in Trolox equivalents (mM). All samples for each assay were performed in duplicate.

### 2.4 Results

#### 2.4.1 HR96 expression

The expression of HR96, a promiscuous nuclear receptor (Karimullina et al., 2012), was examined by qPCR in < 2 to 14 day-old daphnids. qPCR demonstrates that HR96 is expressed throughout a daphnid’s lifespan at nearly equal levels at all of the ages tested; < 2, 4, 7, and 14-days old (Appendix A-1). Therefore, neonatal daphnids have the necessary HR96 to transcriptionally respond to specific toxicant stressors and neonates.
can be used in subsequent acute toxicity tests to determine if compounds that interact with HR96 can modify toxicity.

2.4.2 Single chemical toxicity tests

Forty-eight hour acute toxicity tests were performed to determine concentration ranges for subsequent mixture toxicity tests. All of the unsaturated fatty acids and cholesterol had acute toxicities ranging from 2.90 – 14.4 µM. Cholesterol was the most toxic (LC50 = 2.90 µM), followed by DHA (LC50 = 5.55 µM), LA (LC50 = 10 µM), oleic acid (LC50 = 10.2 µM) and α-linolenic acid (LC50 = 14.4 µM). Of the environmental toxicants, triclosan was the most toxic chemical tested (LC50 = 0.835 µM) and atrazine was the least toxic chemical tested (LC50 = 78 µM) (Figure 2.1).

Figure 2.1: Acute toxicity and dose-response curves of select xenobiotic and fatty acid HR96 modulators to *D. magna*. Acute toxicity assays were performed with < 24 hour old daphnids with several endobiotic and xenobiotic chemicals. Dose-response curves were determined using GraphPad Prizm 4.0 and data are provided as mean ± 95% confidence intervals (n = 5 per treatment). x-axis is in logarithmic scale.
2.4.3 Mixtures with LA mimic independent joint action

We hypothesized that HR96 inhibitors may block HR96 activation and this would increase toxicity compared to model estimations. Therefore, daphnids were exposed to the HR96 inhibitors, triclosan or DHA concomitantly with LA (2-10 µM), a HR96 activator. The toxicity of the mixtures increased in a concentration-dependent manner for each of the chemicals tested (Figure 2.2). Chemical toxicity was also compared to the CATAM model used to predict toxicity based on independent joint action (Rider and LeBlanc, 2005). Some of the lower concentrations or single chemical assays showed less toxicity than expected. However, in general, the toxicity estimated from the CATAM independent joint action model was within the 95% Confidence Intervals determined from the acute toxicity tests (Figure 2.2). Originally, we hypothesized that exposure to DHA and triclosan will increase the toxicity of LA in a synergistic fashion. Therefore, our hypothesis is null as the HR96 inhibitors did not repress toxicant acclimation in vivo and cause enhanced toxicity of LA.
Figure 2.2: The toxicity of Linoleic acid (LA) is accurately predicted in a two-chemical mixture containing an HR96 inhibitor (triclosan or DHA). Acute toxicity tests were performed with low (A), medium (B) and high (C) concentrations of triclosan coupled with multiple concentrations of LA, or low (D), medium (E), and high (F) concentrations of DHA coupled with multiple concentrations of LA. Data are provided as mean ± 95% confidence intervals (n = 10). Statistical significance was determined by ANOVA followed by Tukey’s multiple comparison test (*p < 0.05, **p < 0.01, ***p<0.001) Letter ‘b’ represents a statistical difference in comparison to 2 µM LA, whereas the other statistically significant data is in comparison to the control group. The independent joint action model from CATAM was used to predict daphnid survival under mixture conditions with the average 95% confidence intervals from the toxicity tests used to predict variance in the model. The independent joint action model prediction is shown as a line in each of the graphs.
2.4.4 Atrazine provides protection from HR96 inhibitors

Daphnids were also exposed to the HR96 inhibitors, triclosan or DHA concomitantly with atrazine, a HR96 activator. At low concentrations of atrazine, the CATAM model accurately predicts toxicity in daphnids during co-exposures to either triclosan (Figure 2.3ABC) or DHA (Figure 2.3DEF). However at atrazine concentrations ranging from 20 to 80 µM with 40 µM showing the greatest protection, the toxicity of triclosan and DHA decreased. This is neither consistent with typical concentration-dependent toxicity nor consistent with the CATAM model. Instead, a concentration-dependent protective effect was observed (Figure 2.3). This data is not consistent with our hypothesis that HR96 inhibitors would block the ability to adapt to toxicant stress. Conversely, significant concentrations of the HR96 activator, atrazine, are able to induce a protective effect.
Figure 2.3: The HR96 activator, atrazine induces a concentration-dependent interaction that protects daphnids from toxicity induced by triclosan or DHA. Acute toxicity tests were performed with low (A), medium (B) and high (C) concentrations of triclosan coupled with multiple concentrations of atrazine, or low (D), medium (E), and high (F) concentrations of DHA coupled with multiple concentrations of atrazine. Data are provided as mean ± 95% confidence intervals (n = 10 per treatment). Statistical significance was determined by ANOVA followed by Tukey’s multiple comparison test (*p < 0.05, **p < 0.01, ***p < 0.001.) Statistically significant data is in comparison to the control group. The independent joint action model from CATAM was used to predict daphnid survival under mixture conditions with the average 95% confidence intervals from the toxicity tests used to predict variance in the model. The independent joint action model prediction is shown as a line in each graph.
2.4.5 Atrazine induces anti-oxidant protection

Because atrazine is a HR96 activator and HR96 regulates the expression of a suite of drug metabolism enzymes (King-Jones et al., 2006) much like CAR and PXR (Hernandez et al., 2009), we hypothesized that HR96 induced crucial phase II enzymes necessary for the detoxification of triclosan such as glucosyltransferases (Wu et al., 2010). A [14C]testosterone glucosyltransferases assay was performed to test whether atrazine could have induced this crucial triclosan detoxification pathway. However, we did not observe an increase in glucosyltransferase activity (Figure 2.4).

![Graph of testosterone glucosylation activity in atrazine exposed daphnids. Data are provided as mean ± SEM (n = 5). Statistical significance was determined by ANOVA followed by Tukey’s multiple comparison test with p < 0.05 considered significant.](image)

**Figure 2.4: Testosterone glucosylation activity in atrazine exposed daphnids.** Data are provided as mean ± SEM (n = 5). Statistical significance was determined by ANOVA followed by Tukey’s multiple comparison test with p < 0.05 considered significant.
Triclosan toxicity is often associated with the formation of reactive oxygen species (Binelli et al., 2009; Riva et al., 2012). Therefore, we also examined the potential for increased antioxidant defense or decreased ROS after atrazine exposure with three different assays (Figure 2.5). TBARS is a measure for monitoring lipid peroxidation (Armstrong and Browne, 1994). TBARS/daphnid was increased in the atrazine + DHA group relative to the untreated (control), triclosan, and triclosan plus atrazine groups (Figure 2.5A). Atrazine (40 µM) co-exposed with DHA, which is thought to be protective, increased TBARs, indicating that DHA at 8 µM increases lipid peroxidation in the presence of atrazine. Interestingly, chemical treatments with triclosan did not produce significant increases in lipid peroxidation when compared to the DHA + atrazine co-treatments and lipid peroxidation was at UT levels in the presence of 40 µM atrazine + 0.75 µM triclosan.

Atrazine did increase GST activity in *D. magna* with the other treatment groups showing significantly lower CDNB activity (Figure 2.5B). In addition, the TROLOX antioxidant assay kit that measures the cooperativity of several different antioxidant systems, including glutathione peroxidase, superoxide dismutase, catalase, α-tocopherol, ascorbic acid, and glutathione and converts them to TROLOX equivalents demonstrates that atrazine can induce the overall antioxidant defense system of the daphnid (Figure 2.5C). None of the other treatment groups increased TROLOX equivalents. These results are consistent with atrazine providing protection from a wide range of toxicants that induce ROS, including triclosan and unsaturated fatty acids predisposed to lipid peroxidation, through increased antioxidant defenses (Figure 2.5BC).
Figure 2.5: Measurement of GST activity, antioxidant capacity and ROS (TBARS) induced by chemicals and chemical mixtures. Alteration of ROS and protective enzymes by HR96 modulators were determined using the following measurements (A) TBARS assay for lipid peroxidation (B) GST activity determined by CDNB and (C) TROLOX assay for antioxidant mechanism. Data are provided as mean ± SEM (n = 4). Statistical significance was determined by ANOVA followed by Tukey’s multiple comparison test with an ‘a’ referring to groups that are different from UT, ‘b’ referring to groups that are different from DHA+Atrazine, and ‘c’ referring to groups different from atrazine treatments. Asterisks indicate the level of statistical significance (*p < 0.05, **p < 0.01).
2.5 Discussion

We hypothesized that HR96 inverse agonists such as triclosan or DHA would block HR96-mediated transcription by atrazine or LA and increase toxicity by blocking detoxification responses. Neither triclosan nor DHA showed any increased mixture toxicity relative to the CATAM model. This indicates that neither triclosan nor DHA significantly perturbs detoxification responses, or the responses blocked by these chemicals are not involved in the detoxification of atrazine or LA. Instead our data demonstrates that the HR96 activator atrazine provides concentration-dependent protection from other chemicals such as the HR96 inhibitors DHA and triclosan (Figure 2.3). Atrazine’s protection is consistent with CAR- or PXR-like induction of detoxification enzymes and transporters that mediate protection from drugs and environmental toxicants (Wei et al., 2000; Mota et al., 2010).

Triclosan contains a hydroxyl group at the 2’ position. Therefore, in mammals, glucuronidation is the most common pathway for triclosan detoxification (Wu et al., 2010). We hypothesized that atrazine was decreasing triclosan toxicity through induction of glucosidation. However, we did not observe increased testosterone glucosidation (Figure 2.4), indicating that increased glucosidation was not responsible for atrazine’s concentration-dependent protection. We attempted to measure triclosan glucosidation directly through HPLC (Wu et al., 2010), but were unsuccessful in the neonatal daphnids. Next, we considered that triclosan and DHA may be increasing the production of reactive oxygen species (ROS). Triclosan’s mode of action is primarily through increasing ROS and causing DNA damage (Binelli et al., 2009). Atrazine did induce GST activity and
overall anti-oxidant defenses as shown by the TROLOX and CDNB assays (Figure 2.5). Taken together, our data suggests that atrazine acts to protect daphnids from DHA and triclosan, two chemicals that can induce ROS, by inducing anti-oxidant defenses.

Anti-oxidant defenses are regulated by a number of transcription factors such as CAR, PXR, HR96, NF-kB, and Nrf2 (King-Jones et al., 2006; Hernandez et al., 2009; Zhao et al., 2010). Several GST members are regulated by CAR and PXR in vertebrates, and HR96 induces GSTs and carboxylesterases in *D. melanogaster* in response to xenobiotic stress, including phenobarbital (King-Jones et al., 2006). The transcription factors involved in the regulation of GSTs in *D. magna* have not been investigated. However phenobarbital has been shown to increase GST activity and protein levels in *D. magna* in previous studies (Baldwin and LeBlanc, 1996), indicating a potential role of HR96.

The role of HR96 in the regulation of xenobiotic metabolism has been demonstrated in fruitfly (*D. melanogaster*) (King-Jones et al., 2006) and in part in *D. pulex* (Karimullina et al., 2012). *D. magna’s* HR96 is 96% identical to *D. pulex* in the LBD and 98% in the DBD suggesting that *D. magna’s* responses are similar to those of *D. pulex* (Litoff et al., 2014). However, we do not have HR96-null *Daphnia* and the long-term utilization of siRNAs with free swimming *D. magna* has not been examined thoroughly (Kato et al., 2011; Hiruta et al., 2013). Therefore, we do not have proof that the daphnids are responding to atrazine through HR96 in vivo. The transcription factor Nrf2 is also known to exert protective roles against injuries from oxidative stress (Chen and Kong, 2005; Zhao et al., 2010) as its regulates the expression of several antioxidant
and cytoprotective genes (Itoh et al., 1999; Ishii et al., 2000). Nrf2 has been shown to regulate the expression of NQO and GST in mice (Itoh et al., 1997; McMahon et al., 2001) and humans (Venugopal and Jaiswal, 1996). A Nrf2 ortholog has been found within the D. melanogaster genome (CG43286; cnc), and recent research indicates that it is crucial in response to ROS (Pickering et al., 2013). A BLAST search of the D. pulex genome indicates the presence of a Nrf2 ortholog, the link for which has been provided: jgi|Dappu1|312770|NCBI_GNO_0700043. Therefore, atrazine could be inducing its protective mechanisms through Nrf2 or in conjunction with HR96 as data indicates that both transcription factors regulate antioxidant defenses.

Our results demonstrated that at higher concentrations of atrazine, toxicity due to HR96 inhibitors decreased. This concentration-dependent protection failed to fit the CATAM model. This protective effect was consistent at concentrations of approximately 20 – 80 µM, indicating that atrazine must reach levels that are high enough to activate a specific biochemical process. These concentrations of atrazine are not environmentally relevant; however, the purpose of our research was to investigate D. magna responses to antagonistic mixtures of chemicals; in this case HR96 modulators (Karimullina et al., 2012). Atrazine may prove to be a key positive control for activation of HR96 similar to high doses of phenobarbital, TCPOBOP, or dexamethasone that are often used reference compounds or positive controls for activation of CAR or PXR (Kawamoto et al., 1999; Pascussi et al., 2000; Tzameli et al., 2000). The expression of HR96, a NR orthologous to CAR, PXR, and VDR may explain D. magna’s ability to respond to toxicants in a similar manner to mammals (King-Jones et al., 2006; Hernandez et al., 2009).
A mixture of chemicals that act additively or synergistically may reach the necessary concentrations to induce similar measured stress response pathways. For example, propazine, simazine and other triazine herbicides may act alone or additively with atrazine and other dietary and anthropogenic chemicals to activate HR96 (Baldwin and Roling, 2009) The subsequent transcriptional responses may lead to perturbed regulation of detoxification enzymes or other processes. In turn, unintended consequences may occur, not just mixture effects such as response addition, but also antagonistic effects as observed here, or potentially synergistic or potentiation as well.

In summary, HR96 inhibitors did not block the ability of atrazine, an HR96 activator, to help daphnids respond to toxicant or fatty acid stress. Instead, atrazine appears to have activated detoxification responses, probably through HR96 but also potentially through other transcription factors, which induced a concentration-dependent protective response. This work demonstrates that atrazine and other chemicals may have unique mixture effects on aquatic invertebrates such as daphnids if their concentrations or responses are strong enough leading to drug-toxicant and toxicant-toxicant interactions (Delgoda and Westlake, 2004; Hernandez et al., 2009).

2.6 References


Ginjupalli, G.K., Baldwin, W.S., 2013. The time- and age-dependent effects of the juvenile hormone analog pesticide, pyriproxyfen on Daphnia magna reproduction. Chemosphere 92, 1260-1266.


- 51 -


3.1 Abstract

Acclimating to toxicant stress is energy expensive. In laboratory toxicology tests dietary conditions are ideal, but not in natural environments where nutrient resources vary in quality and quantity. We compared the effects of additional lipid resources, docosahexaenoic acid (n-3; DHA) or linoleic acid (n-6; LA), or the effects of the toxicants, atrazine or triclosan on post-treatment starvation survival, reproduction, and lipid profiles. Chemical exposure prior to starvation had chemical-specific effects as DHA showed moderately beneficial effects on starvation survival and all of the other chemicals showed adverse effects on either survival or reproduction. Surprisingly, pre-exposure to triclosan inhibits adult maturation and in turn completely blocks reproduction during the starvation phase. The two HR96 activators tested, atrazine and LA adversely reduce post-reproduction survival 70% during starvation and in turn show poor fecundity. DHA and LA show distinctly different profiles as DHA primarily increases the percentage of large (>37 carbon) phosphatidylcholine (PC) species and LA primarily increases the percentage of smaller (<37 carbon) PC species. The toxicants atrazine and triclosan moderately perturb a large number of different phospholipids including several phosphatidylethanolamine species. Some of these polar lipid species may be biomarkers for diets rich in specific fatty acids or toxicant classes. Overall our data demonstrates that toxicants can perturb lipid utilization and storage in daphnids in a chemical specific
manner, and different chemicals can produce distinct polar lipid profiles. In summary, biological effects caused by fatty acids and toxicants are associated with changes in the production and use of lipids.

3.2 Introduction

Acclimation to toxicants requires significant energy. The advent of global gene expression technologies has provided unique insights demonstrating numerous energy metabolism changes that occur following toxicant exposure. For example, Cr(VI) increases liver and heart-type FABP and glucose transporter, GLUT2, while repressing apolipoprotein B and cytochrome c oxidase expression in *Fundulus heteroclitus* liver (Roling et al., 2006). Carbon tetrachloride exposure leads to induction of genes related to phospholipid structure, lipid metabolism, and glycolysis in *Oncorhynchus mykiss* (Koskinen et al., 2004). Recent studies have shown that polyaromatic hydrocarbons and polychlorinated biphenyls disrupt mitochondrial function and oxidative phosphorylation in *F. heteroclitus* hepatocytes (Du et al., 2015), and the flame retardant Firemaster 550 (FM550), alters nutritional status in *Daphnia magna* by perturbing fatty acid, glucose, and amino acid metabolism (Scanlan et al., 2015).

Interestingly, toxicant responsive transcription factors such as AhR, CAR, and PXR that regulate phase I-III detoxification also regulate nutrient allocation and energy homeostasis (Ueda et al., 2002; Dong et al., 2009; Hernandez et al., 2009; Lu et al., 2015). For example, the AhR agonist TCDD alters the expression of genes involved in cholesterol biosynthesis, lipogenesis, and glucose metabolism (Sato et al., 2008).
Phenobarbital activation of CAR down-regulates fatty acid oxidation and glucose synthesis, and reduces thyroid hormone activity by increasing thyroid hormone metabolism (Ueda et al., 2002; Maglich et al., 2004). PXR activation by PCN down-regulates β-oxidation and ketogenesis, and increases lipogenesis (Nakamura et al., 2007). CAR and PXR also cross talk with insulin or glucagon responsive transcriptions factors such as forkhead box O1 (regulates gluconeogenesis and glycogenolysis by insulin signaling), forkhead box A2 (regulates β-oxidation and ketogenesis), cAMP-response element binding protein (involved in gluconeogenesis), and peroxisome proliferator activated receptor gamma coactivator 1α (induces genes involved in mitochondrial oxidative metabolism) (Konno et al., 2008; Gao and Xie, 2012).

*Daphnia* and *Drosophila* HR96 receptors are orthologous to CAR/PXR/VDR receptors in mammals (Litoff et al., 2014), and aid in acclimation to toxicant stress (King-Jones et al., 2006; Sengupta et al., 2015). *Drosophila* HR96 has also been shown to regulate gastric lipase (Magro), Niemann Pick type C1, Acyl coenzyme A acyltransferase, and ABC transporter genes involved in cholesterol and triacylglycerol homeostasis and transport (Bujold et al., 2010; Sieber and Thummel, 2012). DHA represses both mammalian CAR and *D. magna* HR96 activity (Li et al., 2007; Lu et al., 2008; Karimullina et al., 2012), and linoleic acid (LA, n-6 fatty acid) activates both CAR and HR96 (Finn et al., 2009; Karimullina et al., 2012), indicating similarities between the mammalian and invertebrate receptors in their response to some lipids. In addition, triclosan, a commonly used antimicrobial agent in household products, represses HR96 activity, and atrazine, a triazine herbicide activates HR96 (Karimullina et al., 2012).
Atrazine is known to cause adverse mixture responses with other pesticides (Pape-Lindstrom and Lydy, 1997; Chen et al., 2015), however, it may also provide protection from triclosan and unsaturated fatty acids in *D. magna* by inducing enzymes that provide protection from lipid peroxidation (Sengupta et al., 2015). Because oral exposure to toxicants and PUFAs may occur simultaneously, there may be competition for receptors. Therefore, toxicants may cause inappropriate lipid-like responses or alter HR96’s ability to respond to diet. Conversely, exposures to specific or changing diets may perturb an individual’s toxicant responses and chemical sensitivity (Karimullina et al., 2012; Ginjupalli et al., 2015; Leão et al., 2015).

PUFAs are crucial structural and regulatory lipids within phospholipid membranes. Alterations in dietary PUFA levels, n-3 fatty acids and n-3/n-6 ratio can also affect membrane-linked cellular processes involved in energy metabolism (Hulbert et al., 2005). Research indicates that changes in dietary lipids impact metabolic rate, neuropeptide activity, second messenger generation and gene expression thus affecting several glucose and lipid metabolism pathways; confirming the role of PUFAs as signaling molecules related to growth, development, and energy metabolism (Hulbert et al., 2005; Wei et al., 2010; Catala, 2013).

PUFA-rich phytoplankton are associated with better growth rate and fecundity in both invertebrates and vertebrates (Verreth et al., 1994; Brett and Muller-Navarra, 1997). PUFAs such as arachidonic acid (n-6 fatty acid), eicosapentaenoic acid (EPA, an n-3 fatty acid) and DHA (n-3) are considered important in the growth and reproduction of aquatic species, including arthropods (Brett and Muller-Navarra, 1997; Ginjupalli et al.,
2015). *D. magna* growth may be directly attributed to increases in n-3 fatty acids such as α-linolenic acid, DHA and EPA; where α-linolenic acid is considered the primary n-3 producer and EPA is considered the primary final n-3 product (Becker and Boersma, 2005). *D. magna* is known to retain EPA and arachidonic acid during starvation or when food is scarce (Brett et al., 2006). Some studies indicate DHA is retained in the ovaries of daphnids (Bunescu et al., 2010), but most research indicates that DHA is rapidly converted to EPA in daphnids (Taipale et al., 2011).

Dietary restriction positively effects lifespan in some species (mice, *C. elegans*, *D. melanogaster*), however positive impacts of diet restriction are not prevalent in all species (Mair and Dillin, 2008; Latta IV et al., 2011). Under limited diet resources organisms tend to reduce reproduction because of its metabolic costs and invest in somatic maintenance for better survival until normal conditions are available for reproduction. This also explains the lifespan-extension observed in several organisms under dietary restriction (Mair and Dillin, 2008). *D. magna* is a suitable model for understanding life history trade-offs of dietary restriction as there is already a vast amount of literature available on relationship between food concentrations and daphnid life history strategies (Latta IV et al., 2011). There is also an increasing interest in understanding how diet plays a role in determining the toxicant sensitivity of different organisms (Pavlaki et al., 2014). For example, algal diet and lipid concentrations have been proposed to be confounding variables in pesticide-induced male production (Olmstead and LeBlanc, 2003; Ginjupalli et al., 2015). In a natural ecosystem, organisms are exposed to different kinds of diets at different times of the year. One such example is
algal succession in freshwater ecosystems, which has an impact on the higher organisms of the food chain. This variation has the potential of acting as a biotic stressor as the diet may interact differently with key lipid receptors. It is important to understand interactions between stressors, and Daphnia with its well understood ecology, provides an excellent model for linking ecology or physiology to specific metabolic and genomic changes (Colbourne et al., 2011).

Our goal is to determine if environmental toxicants that perturb HR96 activity could alter resource allocation in D. magna. In part, we want to determine if D. magna would alter its resource allocation towards survival or reproduction during starvation stress depending on the chemicals to which they had been previously exposed, and test whether specific polar lipids are associated with specific chemicals or physiological responses. We tested whether lipids (DHA or LA) or toxicants (atrazine or triclosan) may alter resource allocation through starvation survival, starvation reproduction and polar lipid profiles.

3.3 Materials and Methods

3.3.1 Daphnia magna culture:

D. magna were maintained at 21-23°C in moderately hard water at a pH of 8.1 – 8.3 in a 16:8 light:dark cycle and fed cultured Pseudokirchneriella subcapitata (Aquatic Biosystems, Fort Collins, CO USA) supplemented with TetraFin fish food (Masterpet Corp., New South Wales, Australia) as described previously (Ginjupalli and Baldwin, 2013).
3.3.2 Chemicals:

LA (≥ 99 %), DHA (≥ 98 %) and triclosan (97%) (Sigma-Aldrich, St. Louis, MO USA) stock solutions were dissolved in absolute ethanol (Sigma-Aldrich Chemical Co., Inc, Milwaukee, WI USA). Atrazine (98.9%) (Sigma-Aldrich, St. Louis, MO USA) stock solution was dissolved in 99.7% DMSO (Fisher Scientific, Fair Lawn, NJ, USA).

3.3.3 Chronic toxicity tests

The reproductive toxicity of DHA, LA, atrazine and triclosan were determined by exposing <24-h neonates (n=10) to these chemicals at multiple concentrations in 40 ml of culture medium that was renewed every other day for 21 d (ASTM, 1988; Baldwin et al., 2001). Daphnids were fed 3 x 10⁶ P. subcapitata cells supplemented with 50 µl of an aqueous suspension of blended, TetraFin fish flakes at 2.5 mg/ml dry weight 2X per day as described previously (Baldwin et al., 1997; Ginjupalli et al., 2015). Chemical concentrations used in the chronic toxicity tests were determined based on previously published acute toxicity tests performed in our laboratory (Sengupta et al., 2015). Atrazine was reconstituted in 99.7% DMSO for a total of 0.016% DMSO in each exposure beaker including the solvent-only (untreated; UT) group. DHA, LA and triclosan were reconstituted in 100% ethanol for up to a total of 0.004% ethanol in each exposure beaker including the UT group. Differences in reproduction were determined by one-way ANOVA followed by Fisher’s Least Significant Difference as the post-hoc test with a p-value of 0.05 considered significant. Statistical analysis was performed using GraphPad Prism 6 (GraphPad Software, San Diego, CA USA).
3.3.4 Starvation assay:

*D. magna* (<24 h old) were fed and exposed to chemical treatments, LA (4 and 8 µM), DHA (2 and 4 µM), atrazine (20 and 40 µM) and triclosan (0.25 µM) (n=12) as described above. On day 5, chemical exposure and feeding was stopped (Figure 3.1). Starvation survival and reproduction in the adolescent daphnids was monitored up to day 13 as none of the animals survived past day 13. Differences in reproduction were determined by one-way ANOVA with a general linear model followed by Fisher’s Least Significant Difference test with a p-value of 0.05. Differences in survival were determined by Fisher’s exact test.
Figure 3.1: Outline of the experimental design and timeline for the treatments and starvation of daphnids as described in the Materials and Methods.

3.3.5 Cholesterol analysis:

Treated and untreated daphnids were collected and homogenized in 100 µl of a pH 7.4 0.01M HEPES, 0.05M EDTA, 10% glycerol buffer. Tubes were centrifuged at 10,000 rpm for 1 minute, and supernatant cholesterol concentrations were determined using a
commercially available fluorometric assay kit (Cayman Chemical Co., Ann Arbor, MI USA) at an excitation wavelength of 550 nm and an emission wavelength of 590 nm (Amundson and Zhou, 1999).

3.3.6 Lipid extraction and Lipidomic analysis:

Daphnids were collected at 5-days old (prior to starvation; see Figure 3.1) for lipid extraction and lipidomic analysis. Polar lipids were extracted from 5 tubes of 5 juvenile daphnids using previously published protocols (Fitzgerald et al., 2007) adapted to *D. magna*. Daphnids were homogenized and shaken in a 1 ml, 1:1 mix of water:chloroform. Following layer separation, 1.5ml of a 1:2 solution of chloroform:methanol was added and vortexed. The solution was centrifuged for 1 minute at 600 rpm, and the lower layer was collected using a glass Pasteur pipette. Chloroform addition (0.5 ml) followed by vortexing, centrifugation, and collection was performed two more times. The lipid extract was cleaned with subsequent washes of 1M KCl (0.5ml) and water. The lower layers were collected, placed in a teflon covered glass vial and dried under nitrogen. Samples were then frozen with dry ice and sent to the Kansas Lipidomics Research Center for lipidomic analysis. Lipid profiles were determined by mass spectrometry (electrospray ionization triple quadrupole mass spectrometer from Applied Biosystems API 4000) at Kansas Lipidomics Research Center as described previously (Isaac et al., 2007).

Changes in overall lipid profiles were determined by two-way ANOVA with repeated measures and the Bonferonni post-hoc test. One-way ANOVA followed by
Tukey’s post-hoc test was used to investigate differences between treatment groups for each of the individual polar lipid species. In addition, hierarchical clustering combined with one-way ANOVA with a p-value cut-off of 0.01 was used to cluster and visualize significant changes in the concentrations of individual lipids with MultiExperiment Viewer. PCA was performed with SAS 9.3 to show and confirm associations between specific lipids and chemical treatments (SAS Institute Inc., Cary, NC USA).

3.4 Results

3.4.1 Reproductive toxicity during 21-day chemical exposures

Reproductive fitness was determined in *D. magna* exposed to atrazine, triclosan, DHA, and LA. DHA, LA and triclosan did not reduce reproduction at any of the concentrations tested. However, atrazine reduced reproduction by 50% - 90% at concentrations from 10 - 80 µM (Appendix B-1) with little effect (14%) at 5 µM. Previous studies found that atrazine reduced reproduction almost 30% at 2.32 µM (0.5 mg/L) and greater than 80% at 69.55 µM (15 mg/L) (Palma et al., 2009). Therefore, our data for atrazine is similar but not identical to what has been previously reported. We observed a concentration-dependent drop in reproduction during triclosan exposures from 0.022 – 0.345 µM, but it was not statistically significant by ANOVA. Previous studies reported 0.22 µM (64 µg/L) triclosan reduced reproduction during chronic toxicity tests with *D. magna* (Peng et al., 2013). To our knowledge, this is the first paper to report the reproductive toxicity of DHA or LA (Appendix B-1).
Subsequent research with each chemical was performed at concentrations that did not significantly alter reproduction based on our chronic toxicity tests for DHA (2-4 μM), LA (4-8 μM), and triclosan (0.25 μM). We continued to use 20 – 40 μM atrazine despite the fact these concentrations reduced fecundity because these concentration have been shown to protect *D. magna* from the toxic effects of other chemicals such as triclosan and DHA most likely because of HR96 activation (Sengupta et al., 2015).

### 3.4.2 Chemical-induced changes in starvation survival and fecundity in *D. magna*

We hypothesized that several endogenous and xenobiotic chemicals, especially those that increase HR96 activity, may perturb resource allocation leading to increased survival during periods of starvation because of increased lipid absorption (King-Jones et al., 2006; Horner et al., 2009). Therefore, daphnids aged 1-5 days old were exposed to either HR96 activators (atrazine, LA) or HR96 inhibitors (triclosan, DHA) (Karimullina et al., 2012) and then starved to determine chemical-induced changes in starvation survival and reproduction. Survival rarely varied between UT daphnids and the other groups with the exception of day 8 and day 11 in which DHA-treated daphnids showed greater survival than untreated daphnids. However, daphnids exposed to DHA did show increased survival relative to daphnids exposed to LA and atrazine (Figure 3.2). In general there was a pattern indicating better survival for DHA > triclosan > LA > atrazine. This is the opposite of what we hypothesized as both HR96 inhibitors (triclosan and DHA) showed better starvation survival compared to the HR96 activators (atrazine and LA).
Reproduction was split into three tiers: groups that reproduced well, those that reproduced moderately well, and those that reproduced poorly during starvation (Table 3.1). The DHA groups and UT group reproduced the best (Table 3.1). Therefore, DHA showed some benefit in both starvation survival and reproduction relative to the other treatment groups (Figure 3.2; Table 3.1). We expected that atrazine would demonstrate poor reproduction based on the chronic toxicity test data (Appendix B-1) and it did;
however, reproduction was even worse in triclosan- and 4µM LA-exposed daphnids. Interestingly, the triclosan-exposed daphnids did not reproduce during starvation and appeared to enter a senescence or quiescence in which they no longer continued to develop. Other treatment groups continued to develop into adults despite the lack of food.

**Table 3.1: Survival of daphnids following release of broods.**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Neonates/Daphnid#</th>
<th>Adults that reproduced</th>
<th>Adults that survived after reproduction</th>
</tr>
</thead>
<tbody>
<tr>
<td>UT</td>
<td>8.83 ± 3.12*</td>
<td>7</td>
<td>7</td>
</tr>
<tr>
<td>2 µM DHA</td>
<td>11.42 ± 1.92²</td>
<td>12*</td>
<td>9</td>
</tr>
<tr>
<td>4 µM DHA</td>
<td>8.92 + 1.29a</td>
<td>12*</td>
<td>11</td>
</tr>
<tr>
<td>4 µM LA</td>
<td>2.17 ± 1.16³</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td>8 µM LA</td>
<td>7.83 + 1.91³³</td>
<td>9</td>
<td>3*</td>
</tr>
<tr>
<td>20 µM Atr</td>
<td>3.83 + 0.99³</td>
<td>8</td>
<td>2*</td>
</tr>
<tr>
<td>40 µM Atr</td>
<td>4.00 + 1.13³</td>
<td>7</td>
<td>2*</td>
</tr>
<tr>
<td>0.25 Tric</td>
<td>0.00 + 0.00³</td>
<td>0*</td>
<td>0</td>
</tr>
</tbody>
</table>

n = 12 per treatment group
# Letters that are different indicate statistical significance from other groups via ANOVA with a general linearized model followed by Fisher’s LSD.
* indicates statistically different from UT group as determined by Fisher’s 2x2 test.

During starvation, several of the adult female daphnids died within 24 hours of releasing a brood (Table 3.1). A significant drop in post-reproductive survival (approaching 70%) was observed in the atrazine and LA treated groups; both HR96 activators. This suggests an allocation of resources towards reproduction and away from
survival in the daphnids treated with these HR96 activators. This is in contrast to triclosan-treated daphnids that did not reproduce and instead remained in the adolescent stage during the entire starvation phase (up to Day 13), and the DHA-exposed daphnids that survived following the release of their first brood.

3.4.3 Chemical exposures alter cholesterol levels and polar phospholipids

HR96 is a potent magro inducer in *Drosophila* (Sieber and Thummel, 2009). Magro, also known as gastric lipase in mammals, hydrolyzes cholesterol esters and promotes cholesterol clearance from the intestine (Sieber and Thummel, 2012). Therefore, we hypothesized that HR96 activators would reduce cholesterol. However, atrazine-exposure increased cholesterol concentrations greater than all other groups and the other HR96 activator, LA, produced the 2nd highest cholesterol concentrations. All chemical exposures increased cholesterol concentrations in the homogenized daphnids relative to control daphnids (Figure 3.3A).

In part because cholesterol did not drop as expected and in part because we suspected that HR96 regulators and lipids alter lipid profiles, we examined changes in several polar lipid classes. Total lipids were significantly lower in toxicant-exposed groups (atrazine and triclosan); reflected primarily by a small drop in PC and larger drop in PE (Table 3.2). To take a closer look at relative levels of individual lipid species, samples were evaluated as percent total signal. PC comprised 75% and PE 15.5% of the lipidome as these two polar lipids make up greater than 90% of the polar lipids in *D. magna*. SM (4.4%), PI (2.8%), LysoPE (0.74%), PS (0.57%), LysoPC (0.52%), PG
(0.44%), and phosphatidic acid (0.08%) comprise the remaining polar lipid species (Figure 3.3B). Again, PC and PE were significantly altered following chemical exposures. Percent total PC was increased in all groups compared to the untreated daphnids, and the percent PC in the toxicant exposures (atrazine, triclosan) was higher than in the PUFA exposures (LA, DHA) (Figure 3.3B). Consequently, percent total PE was reduced about 2X in the atrazine and triclosan groups (Figure 3.3B).
Table 3.2: Quantity of each lipid type found in the polar lipids extracted from treated and untreated *D. magna*.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>UT#</th>
<th>DHA (4µM)</th>
<th>LA (4µM)</th>
<th>Atr (40 µM)</th>
<th>Tric (0.25µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysoPC</td>
<td>0.230±0.068</td>
<td>0.149±0.111</td>
<td>0.125±0.026</td>
<td>0.074±0.032</td>
<td>0.081±0.046</td>
</tr>
<tr>
<td>PC</td>
<td>33.694±7.536</td>
<td>28.955±7.453(^a)</td>
<td>25.524±4.164(^a)</td>
<td>18.552±3.291(^a)</td>
<td>12.805±7.230(^a)</td>
</tr>
<tr>
<td>SM</td>
<td>1.951±0.431</td>
<td>1.114±0.248</td>
<td>1.368±0.319</td>
<td>0.985±0.180</td>
<td>0.669±0.531</td>
</tr>
<tr>
<td>LysoPE</td>
<td>0.327±0.103</td>
<td>0.224±0.053</td>
<td>0.145±0.043</td>
<td>0.069±0.043</td>
<td>0.055±0.056</td>
</tr>
<tr>
<td>PE</td>
<td>7.045±2.102</td>
<td>5.252±1.532</td>
<td>3.517±0.871</td>
<td>2.131±1.507(^b)</td>
<td>1.463±1.771(^b)</td>
</tr>
<tr>
<td>PG</td>
<td>0.205±0.100</td>
<td>0.104±0.051</td>
<td>0.081±0.028</td>
<td>0.057±0.018</td>
<td>0.036±0.018</td>
</tr>
<tr>
<td>PI</td>
<td>1.258±0.337</td>
<td>0.701±0.231</td>
<td>0.723±0.174</td>
<td>0.422±0.067</td>
<td>0.349±0.304</td>
</tr>
<tr>
<td>PS</td>
<td>0.257±0.058</td>
<td>0.179±0.044</td>
<td>0.203±0.039</td>
<td>0.196±0.052</td>
<td>0.152±0.049</td>
</tr>
<tr>
<td>PA</td>
<td>0.038±0.016</td>
<td>0.023±0.006</td>
<td>0.024±0.012</td>
<td>0.015±0.004</td>
<td>0.014±0.009</td>
</tr>
<tr>
<td>Lipids</td>
<td>45.005±10.395</td>
<td>36.700±9.530</td>
<td>29.709±5.206</td>
<td>22.501±4.600(^b)</td>
<td>15.624±9.755(^bc)</td>
</tr>
</tbody>
</table>

#Data presented as mean nmol lipid/sample +/- standard error. Statistical differences determined by two-way ANOVA followed by the Bonferroni post-hoc test (n=5). ‘a’ refers to different from all other treatments, ‘b’ is different from UT, ‘c’ is different from DHA.
Figure 3.3: Cholesterol concentrations and relative polar lipid levels in daphnids following four days of exposure to different chemicals. (A) Cholesterol concentrations in homogenized daphnids. Data are presented as mean ± SEM. Data was analysed by one-way ANOVA followed by Tukey’s multiple comparison test (n=6) (*p<0.05, **p<0.01 and ***p<0.001). An ‘a’ is different from UT and ‘b’ is different from Atr. (B) Relative polar lipid levels from chemically-exposed daphnids. Data were analyzed by two-way ANOVA (with repeated measures) followed by Bonferroni post-hoc test (n=5). ‘a’ is different from UT, ‘b’ is different from DHA, ‘c’ is different from LA, ‘d’ is different from Atr and ‘e’ is different from Tric (p<0.05, *p<0.01, **p<0.001).
Therefore, we investigated the relative percent total signal for the individual PE and PC species (Figure 3.4). Compared to the untreated daphnids there are few changes in the PE species with the exception of the 34 and 36 carbon PE species that are significantly lower in the atrazine and triclosan groups (Figure 3.4A, Appendix B-3). However, there are many changes in individual PC species between UT and chemical exposures (Figure 3.4B) and between the different chemical exposure groups (Appendix B-4). The percent signal for PC 34:2, 36:3, and 36:4 are perturbed in all groups compared to the control, and PC 37:0 is different from the controls in all groups except triclosan. Several large PC species (40:7, 38:5, 37:1) are only different from the controls following DHA exposure. Triclosan specifically perturbed PC 34:4 and 36:6 (Figure 3.4B; Appendix B 4-5).

SM, PI, PE and PS species also showed individual differences relative to controls or other groups (Appendix B-2). SM 38:1 is decreased in the atrazine, triclosan, and DHA groups, PI 36:2/3/4 are increased by several treatments including DHA, PI 34:2 is increased only by DHA, and PS 36:2, and 36:4 are increased by triclosan.
Figure 3.4: Changes in phosphatidylethanolamine (PE) (A) or phosphatidylcholine (B) composition among different exposure groups. Changes in percent signal of individual PE (A) or PC (B) species following chemical exposures (UT = control, DHA 4 = 4 μM DHA, LA 4 = 4 μM LA, Atr 40 = 40 μM atrazine, Tric 0.25 = 0.25 μM triclosan) in daphnids were analyzed by two-way ANOVA (with repeated measures) followed by the Bonferroni post-hoc test. Statistical differences between different groups are noted with letters; ‘a’ indicates UT vs DHA, ‘b’ is UT vs LA, ‘c’ is UT vs Atr, ‘d’ is UT vs Tric, ‘e’ is DHA vs Tric, ‘f’ is LA vs Atr and ‘g’ is LA vs Tric (only letter = p<0.01, * indicates p<0.001).
PCA was performed because two-way ANOVAs (Figure 3.4; Appendix B-2, 4) indicated associations between specific polar lipids and chemical exposures. Specific lipid species are associated with the different treatments. PCA demonstrates that the atrazine and triclosan groups are most similar while the DHA and LA groups often show opposing lipid profiles (Figure 3.5). In addition, PCA confirmed that several large PC species such as 36:1, 37:1, 37:5, 37:6, 38:5, 38:6, 40:7, and 40:8 are associated with DHA exposure. Some SM, PI, and mid-weight PC species such as SM 32:1, 38:1, 40:1, 34:1, PI 36:2, 36:4, 34:2, and PC 36:4, 36:3, 34:2, 35:2 are associated with LA exposure. PS 36:2, 36:4, SM 42:0, 34:1, 36:2, and PC 34:3, 33:3, 32:2, 35:0, 36:6 are associated with triclosan and atrazine exposure.

PCA and two-way ANOVA indicate distinct lipid groupings based on the different chemical exposures (Figures 3.4-3.5). Therefore we performed hierarchical clustering of the different treatments and polar lipids coupled with one-way ANOVA to filter non-significant polar lipids and better show specific patterns of polar lipids (Appendix B-6). The hierarchical clustering confirms the similarities of atrazine and triclosan exposures, and demonstrates a large difference between DHA exposure and the other exposures as DHA clusters into a separate clade from the other exposure groups. Scatter plots also confirm significant changes in specific polar lipid species (Appendix B-3,5,7). For example, DHA caused the most changes in PC species with significant increases in large PC species such as 36:1, 37:0, 37:1, 37:5, 37:6, 38:5, 38:6, 40:7, and 40:8. PC 37:0, 37:1, 38:5, 38:6, 40:7, and 40:8 are either major PC species or show relatively large changes (>2X) following DHA exposure. ANOVA also confirmed the
PCA data indicating that LA and DHA cause opposing actions on the relative abundance of several phospholipids (Appendix B-5, Figure 3.5). The change in concentration of some of these PC species may provide a novel biomarker for DHA or LA exposure or function.

Atrazine and triclosan showed similar lipid profiles. Each altered the relative concentrations of 15 different phospholipids with 8 of the changes being identical in the atrazine/triclosan groups (Appendix B-3,5,7). Most of the polar lipids that show differences between control and triclosan or control and atrazine were within the PCs (Appendix B-5). Overall there were more significant changes in polar lipids caused by the toxicant exposure groups (atrazine and triclosan) in comparison to the control group than the PUFA groups (LA and DHA) compared to the control group; however, changes were usually larger in the PUFA exposed groups.
Figure 3.5: Principle component analysis reveals associations between chemical exposures and lipid profiles in *D. magna*. Variability among polar lipids was observed following chemical treatment.

3.5 Discussion

Each of the chemicals tested induced unique lipidomic signatures in the daphnids (Figure 3.5, Appendix B-6). For example, LA and DHA had its most prominent effects on relative abundance of PC species with LA predominantly effecting PCs 36 carbons or less and DHA predominantly effecting PCs 37 carbons or more (Figure 3.4-3.5; Appendix B-5). In addition, LA and DHA had nearly diametrically opposed placement
following the PCA (Figure 3.5). The two toxicants tested, triclosan and atrazine showed similar signatures to each other (Figure 3.5) primarily because each significantly perturbs eight polar lipids in nearly identical fashion including significant decreases in several PE and PI species (Figure 3.5, Appendix B-3,7). Still triclosan and atrazine showed unique features from each other as atrazine preferentially increased the relative abundance of 36-38 carbon PC species, while triclosan reduced mid-range PS (36:2, 36:4) and PC (34:4, 36:4) species (Appendix B-3,5,7).

It is possible that one or more of these polar lipid species may become biomarkers of exposure to a specific type of chemical, or a biomarker associated with a specific effect. Of special interest is triclosan. Triclosan caused an interesting phenotype during starvation. In the standard 21-day chronic toxicity test, triclosan had no significant effect on reproduction (Appendix B-1). However, triclosan exposure prior to starvation caused starved daphnids to enter a senescent state as the triclosan-exposed daphnids did not develop beyond adolescence and therefore never reproduced. This effect was specific to triclosan only in our study, where all the other groups developed into reproductive adults even in the absence of food. The lack of reproduction following triclosan exposure could have devastating effects on a stressed pond population.

Recent research indicates that triclosan inhibits fatty acid synthase (FASN) (Sadowski et al., 2014) and this may contribute to the repressed development as lipid stores may not have been produced or available for growth and development. Polar lipid concentrations were lower in triclosan exposed daphnids (Table 3.2). However, survival was not lower than the other groups indicating lipids were available for survival, but not
reproduction and development. Several polar lipids were repressed by triclosan exposure including multiple PE species (Figures 3.4-3.5; Appendix B-3). Triclosan (and atrazine) moderately decreased PE 36:2, which has previously been associated with diabetes (Lappas et al., 2015). To a lesser extent some PI, PS, and PC species were also perturbed (Appendix B-3, 5, 7) by triclosan exposure. However none of these lipid species are specific to triclosan as other chemicals that did not cause senescence also perturbed their relative abundance.

Other examples of unique signatures include DHA and LA. DHA’s effects on polar lipids were consistent across all analyses (Figures 3.4-3.5; Appendix B2-B7) with several large PC species highly increased by DHA including PC 37:0, 37:1, 38:6, 40:8 and to a lesser extent 37:5, 37:6, 38:5 (Figures 3.4-3.5; Appendix B-4, 5). The effects of LA on lipid profiles are not as unidirectional for the PC species. Smaller species with one double bond (PC 30:1, 32:1, 34:1) are decreased by LA exposure, and mid-sized PC species with multiple double bonds are primarily increased (PC 34:2, 35:2, 36:3, 36:4) by LA exposure (Figures 3.4-3.5, Appendix B-5). LA has been used to protect embryos during cryogenic freezing and the high production of 36 and 38 carbon PC is hypothesized to be involved in this protective effect (Leão et al., 2015). Interestingly, in humans an increase in PC 36:4, a major supplier of arachidonic acid to the blood (Uhl et al., 2015), is associated with type I diabetes (Wittenbecher et al., 2015). PC 36:4 was increased by LA and atrazine, but decreased by DHA. DHA appears to be protective from diabetes and other metabolic diseases (Wei et al., 2010; Bhaswant et al., 2015), and
atrazine is associated with mitochondrial dysfunction and obesity (Mokdad et al., 2001; Lim et al., 2009).

DHA is the only chemical that improved physiological endpoints measured during starvation relative to controls. Therefore, the reduction of PC 36:4 and the increase in large molecular weight PCs and PE 37:1 by DHA are associated with better starvation survival and reproduction. However, more work needs to be done to better define these associations. A much greater percentage of DHA-exposed daphnids successfully reproduced compared to the other groups (Table 3.1). In most exposure groups, reproduction during the starvation phase of the study often led to death of the reproducing adult, however the DHA-exposed adults showed better survival following their first brood than the other groups (Table 3.1). The higher number of adult daphnids that reproduced and the ability of some DHA-exposed daphnids to produce a second brood are key factors in the increased reproduction in DHA-exposed daphnids.

In contrast, triclosan, atrazine, and LA adversely affected starvation survival or reproduction (Figure 3.2). Only atrazine reduced fecundity during the 21-day chronic toxicity test compared to untreated daphnids (Appendix B-1), therefore the other chemicals clearly show greater toxicity to daphnids under the stressful starvation conditions (Figure 3.2, Table 3.1). Post-reproduction survival was significantly reduced (up to 70 %) by atrazine and LA (both HR96 activators) (Karimullina et al., 2012) exposure during starvation (Table 3.1). Chemical toxicity tests are performed under ideal conditions where temperatures are stable, light-cycles are optimal, and food is plentiful. However, food content may change because of temperature and seasonal changes leading
to algal succession (Schlechtriem et al., 2006; Ginjupalli et al., 2015). These changes may alter the lipid and protein content available (Bergman Filho et al., 2011) and in turn perturb the nutritional content in the algae, altering energy budget, lipid profiles, and potentially increasing chemical toxicity (Pieters et al., 2006). Therefore, chemical toxicity may be greater than expected in natural environments because of seasonal changes that perturb algal succession and nutritional content. Of special concern is triclosan.

The percentage of PC was higher in all exposure groups relative to controls with the triclosan and atrazine groups showing the highest percent PC, 83 and 84.5% respectively. This occurred at the expense of PE (Figure 3.3), and is in part why several PE’s are associated with the UT group (Figure 3.5). DHA and LA have been shown previously to preferentially incorporate into phosphatidylcholines (Bouroudian et al., 1990; Leão et al., 2015). This may help explain the increase in PC following lipid exposures; however, this does not explain the increase in PC in the atrazine and triclosan groups.

The increase in PC may be a protective mechanism as phosphatidylcholines are associated with protection of various organs. An increase in PC is associated with increased survival of cryopreserved embryos (Leão et al., 2015), and reduced liver toxicity following exposure to alcohol, carbon tetrachloride, high-fat diet, and other toxicants primarily associated with oxidative stress (Aleynik et al., 1997; Lieber et al., 1997). However, CCl₄-induced steatosis in rats causes an overall decrease in both hepatic PE and PC with an increase in specific species such a PE 34:1 that is decreased
following LA, atrazine, and triclosan exposure in our study (Figure 3.4; Appendix B-3), and PC 38:5 and 40:7 that are increased in severe steatosis (Ahn et al., 2008) and by atrazine or DHA exposure in our study (Figure 3.4; Appendix B-5).

A recent study with JEG-3 cells demonstrated several changes in lipid profiles following exposure to tributyltin and perfluorinated chemicals including an increase in several PC species; however, few of these changes are similar to what we observed (Gorrochategui et al., 2014). A recent paper with *D. magna* also indicates that tributyltin disrupts PC incorporation into eggs (Jordão et al., 2015). There have been few published studies investigating the effects of toxicants on lipid profiles, and only two previous studies with *D. magna* (Jordão et al., 2015; Scanlan et al., 2015). Therefore, it is difficult to draw conclusions, but overall the data indicates there are clearly distinct lipid profiles between species, tissues, disease states, or mediated by chemical exposures and it appears that the incorporation of more PC into polar lipids may be a protective mechanism.

The two HR96 activators, LA and atrazine showed a similar physiological response with starved animals dying shortly after releasing their first brood. This suggests that their energy resources were put into reproduction and not survival. However, there are few obvious patterns between the two chemicals that associate specific lipids with a decrease in post-reproduction survival with the possible exception of some mid-weight PC (36:3, 36:4, 37:0) and PE (37:1, 38:5) species (Appendix B-3, 5, 6). Similarly, there are no lipids that can be easily associated with triclosan’s inhibitory effects on reproductive maturity. Overall, there are lipid profiles specific to the chemical
exposures; however there are few lipids that can be easily associated with a specific phenotype at this time.

Furthermore, the two HR96 activators do not have similar lipid profiles (Figure 3.5). Similarly, the two HR96 inverse agonists, DHA and triclosan only have a few lipids in common (Figure 3.5, Appendix B-3, 5, 7) such as PE 34:1, 38:5, and PC 36:3, 36:4, suggesting modes of action on lipid profiles are not related to HR96. Interestingly, PI 34:2, 36:2, 36:3, 36:4 are all modulated in a similar pattern by DHA, atrazine, and triclosan (Appendix B-6, 7). PI’s are very important in signal transduction (Kim et al., 2015), but the specific processes altered if any are not known. While we expect incorporation of lipids to vary greatly between toxicants and lipids as DHA and LA can directly incorporate into lipids, this data suggests that HR96 is not mediating many of the lipid profile perturbations observed. In further support of this conclusion, the HR96 inverse agonist, triclosan, and the HR96 activator, atrazine have similar profiles as observed from the PCA (Figure 3.5) and hierarchical clustering (Appendix B-6).

In summary, daphnids exposed to different dietary unsaturated fatty acids or environmental chemicals show diverse and chemical-specific lipid profiles. These profiles or specific lipids may be good biomarkers of exposure or be associated with disease or adverse effects, but these associations are weak or unknown at this time. Lipid profiles are not well associated with HR96 activity. Profiles do show starkly different profiles between the n-6 fatty acid LA and the n-3 fatty acid DHA and similar profiles between the two toxicants, atrazine and triclosan. Chemical exposure also caused unique responses under starvation conditions with DHA providing some survival benefits,
atrazine and LA exposed daphnids that reproduce at the expense of the mother’s survival, and triclosan enhancing survival at the expense of reproduction. This demonstrates that toxicants can alter energy or lipid utilization and storage in daphnids by chemical specific means. Further experiments will attempt to discern developmental differences between neonates and adults that may provide clues as to why triclosan inhibits adult maturation.

In conclusion, chemical exposure causes unique lipid profiles.

3.6 References


Ginjupalli, G.K., Baldwin, W.S., 2013. The time- and age-dependent effects of the juvenile hormone analog pesticide, pyriproxyfen on Daphnia magna reproduction. Chemosphere 92, 1260-1266.


CHAPTER FOUR
EXCHANGE OF POLAR LIPIDS FROM ADULTS TO NEONATES IN *Daphnia Magna*: PERTURBATIONS IN LIPID ALLOCATION BY DIETARY LIPIDS AND ENVIRONMENTAL TOXICANTS

4.1 Abstract

Xenosensing nuclear receptors are also lipid sensors. We hypothesized that toxicant-induced changes in the xenobiotic receptor HR96 would alter lipid profiles and the balance between adult survival and neonate production. Young adult daphnids were exposed to HR96 activators or inhibitors and later starved to test whether exposure altered allocation toward survival or reproduction. The HR96 activators, linoleic acid and atrazine, decreased reproduction as expected with concomitant changes in the expression of the HR96 regulated genes such as magro. The HR96 inhibitors, DHA and triclosan, increased reproduction or neonate starvation survival, respectively but at the expense of maturation. Lipidomic analysis revealed that sphingomyelins (SMs) are predominantly found in neonates and therefore we propose are important in development. DHA and triclosan increased neonatal SM, consistent with HR96’s regulation of Niemann-Pick genes. While DHA altered expression of Niemann-Pick 1b and other HR96-regulated genes as expected, triclosan did not indicating a different mechanism for perturbing SM. Overall, SM appears to be a key lipid in *Daphnia* maturation and further support for this was provided by carmofur, which inhibits sphingomyelin/ceramide metabolism and severely represses *Daphnia* maturation. In conclusion, toxicants can
perturb lipid allocation such as SM metabolism and in turn reduce survival, development and reproduction.

**4.2 Introduction**

Zooplanktons occupy an important place in the aquatic food web because they are able to transfer energy rich molecules (nutrients) from primary producers to higher trophic levels. Seasonal variation may affect nutrient transfer because of changes in the composition of food. For example, algal succession alters nutrient composition in part because different algae species produce different amounts of polyunsaturated fatty acids (PUFAs) (Brown et al., 1997; Hartwich et al., 2012). Accumulation of PUFAs is species specific in the aquatic ecosystem, and eventually this has the potential to alter the PUFA transfer through various trophic levels in the food web (Gladyshev et al., 2011; Hartwich et al., 2013). PUFAs play a direct role in energy metabolism, growth and development through membrane-linked cellular processes and their allocation is tightly regulated (Hulbert et al., 2005; Catala, 2013; Lynn et al., 2015).

Recently, toxicants have been found to perturb allocation of lipid resources. Baillie-Hamilton recognized an increase in rodent weight following treatment with several chemicals and coined the term “metabolic disruptors” as a moniker for chemicals that perturbed the utilization of nutrient-rich resources (Baillie-Hamilton, 2002; LeBlanc et al., 2012). Recent research demonstrates that some chemicals interfere with lipid allocation and cause obesity and related metabolic disorders including non-alcoholic fatty liver disease and type-2 diabetes (Grun and Blumberg, 2009). Another common term for
these chemicals is “obesogens” (Grun and Blumberg, 2009b, a). In addition, acclimating to toxicants can be an energy expensive process (Koskinen et al., 2004; Du et al., 2015) that alters individual demands through behavioral, transcriptional, or metabolic changes (Roling et al., 2006; Chevalier et al., 2015; Du et al., 2015; Sengupta et al., 2016).

Many of these metabolic disruptors perturb transcription factor activity, especially the activity of the nuclear receptors (NRs) that alter lipid utilization and allocation. NRs such as the peroxisome proliferator-activated receptors (PPARs) are activated by endogenous fatty acids and obesogens that increase the depuration of fatty acids from the blood into white adipose tissue or the liver (Lefebvre et al., 2006; Lima et al., 2015). Co-activation of PPARs and its heterodimeric partner retinoid X receptor (RXR) may increase the activity of the obesogen and stimulate beta-oxidation of fatty acids (Ferré, 2004) and in Daphnia magna, activation of RXR was recently shown to perturb nutrient allocation (Jordão et al., 2015). Other NRs are also involved in lipid allocation, including glucocorticoid receptor (John et al., 2016) and farnesoid X-receptor (Zhang et al., 2012). Receptors first considered xenobiotic-sensors such as the constitutive androstane receptor (CAR) (Ueda et al., 2002; Dong et al., 2009), aryl hydrocarbon receptor (AhR) (Lu et al., 2015) and pregnane X receptor (PXR) (Hernandez et al., 2009) also regulate energy balance directly or in part through AMP-kinase (Shindo et al., 2007).

Most of the metabolic disrupting effects of anthropogenic compounds have been investigated in vertebrates, but not extensively studied in invertebrates probably due to a limited knowledge of invertebrate lipid metabolism pathways and their regulation (Jordão et al., 2015). Daphnia and Drosophila HR96 is an ortholog of CAR/PXR/VDR
(Karimullina et al., 2012; Litoff et al., 2014) that regulates phase I-III detoxification genes and mediates energy metabolism through homeostasis and transport of triacylglycerols and cholesterol (King-Jones et al., 2006; Sieber and Thummel, 2012). HR96 is also a key regulator of the Niemann Pick type C gene family involved in cholesterol and fatty acid homeostasis (especially sphingolipids). Niemann Pick disease is a lysosomal storage disorder caused by the improper retention of sphingomyelin and several Niemann Pick genes are sphingomyelinases or sphingomyelin carriers (Horner et al., 2009). The metabolism of sphingomyelins (SM) is important in cell signaling and development and often regulated by its metabolites, ceramides, sphingosine, or sphingosine-1-phosphate (Hannun and Obeid, 2008; Kitatani et al., 2008). In a recently published study with Daphnia magna, we show by principle component analysis (PCA) that chemicals that alter HR96 in adolescent daphnids such as linoleic acid (LA) (n-6 fatty acid), triclosan, or atrazine increased retention of some SM, while others such as docosahexaenoic acid (DHA) increased retention of phosphatidylcholines (PC). Interestingly, triclosan also repressed reproduction or caused a senescent state that stunted maturation of adolescents to adults (Sengupta et al., 2016). Therefore, perturbation of HR96 activity and subsequent changes in SM concentrations may have sublethal effects on resource allocation or effects on development, maturation and reproduction.

We are interested in testing whether exposure to HR96 activators increase lipid uptake and storage in D. magna leading to better starvation survival in lieu of reproduction in comparison to animals exposed to HR96 inhibitors or untreated daphnids.
In addition, we will determine whether there are differences in the allocation of polar lipids between neonates and adults and whether DHA, LA, atrazine, or triclosan perturb the patterns of polar lipids produced and whether these changes are associated with reproductive fitness or development.

4.3 Materials and Methods

4.3.1 Daphnia magna culture

*D. magna* were maintained in culture at 21-23°C in a 16:8 light:dark cycle in moderately hard water. Daphnids were fed *Raphidocelis subcapitata* (Aquatic Biosystems, Fort Collins, CO USA) supplemented with TetraFin fish food (Masterpet Corp., New South Wales, Australia) as described previously (Ginjupalli and Baldwin, 2013). *R. subcapitata* is formerly known as *Pseudokirchneriella subcapitata*.

4.3.2 Chemicals

Atrazine (98.9%) (Sigma-Aldrich, St. Louis, MO USA), GW4869 (Cayman Chemical, Ann Arbor, MI, USA) and carmofur (Abcam Inc., Cambridge, MA, USA) stock solutions were dissolved in 99.7% DMSO (Fisher Scientific, Fair Lawn, NJ, USA). Linoleic acid (LA)(≥ 99 %), docosahexaenoic acid (DHA)(≥ 98 %), palmitic acid (PA)(99%) and triclosan (97%) (Sigma-Aldrich, St. Louis, MO USA) stock solutions were dissolved in absolute ethanol (Sigma-Aldrich Chemical Co., Inc, Milwaukee, WI USA).
4.3.3 Starvation survival - Reproduction assay

From day 1-7, daphnids were fed $3 \times 10^6$ *R. subcapitata* and from day 7 forward, daphnids were fed $3 \times 10^6$ *R. subcapitata* 2X per day until the starvation phase. Algae was supplemented with 50 µl of an aqueous suspension (2.5 mg/ml) of blended TetraFin fish flakes during the feeding period. In our laboratory at 21-23°C, *D. magna* embryos descend into their brood chamber at around day 7 with the first brood release occurring on day 9-10. Therefore, we exposed 7-day old *D. magna* to the fatty acids LA (4 and 8 µM), DHA (2 and 4 µM), and PA (2 and 4 µM), and the xenobiotics atrazine (20 and 40 µM) and triclosan (0.1 and 0.25 µM) (n=12) for four days in a similar manner (but different age) as described previously (Sengupta et al., 2015) and explained pictorially in Figure 4.1. Chemical concentrations were determined based on chronic toxicity tests (Sengupta et al., 2015) or atrazine mediated concentration-dependent protection from triclosan and DHA toxicity (Sengupta et al., 2015). All treatments and the control group were provided a combination of 0.016% DMSO and 0.004% ethanol as solvent matched controls. On day 11 chemical exposures and feeding was stopped and daphnids were starved to determine if fatty acids or toxicants altered allocation of resources towards survival or reproduction. Starvation survival and reproduction in the adult daphnids was monitored until day 20 as none of the animals survived past day 20. In addition, on day 16, neonates were collected from the adults in each treatment and starvation survival of these neonates was followed for 14 days to determine if fatty acids or toxicant exposure caused a change in energy resources within the neonates (n=12). Differences in adult and neonate survival were determined by Fisher’s exact test. Differences in reproduction were
determined by one-way ANOVA with a general linear model followed by Fisher’s Least Significant Difference (LSD) (p-value ≤ 0.05).

4.3.4 Cholesterol and triacylglyceride (TAG) analysis

Seven-day old daphnids were treated with DHA, LA, atrazine and triclosan for four consecutive days (Fig. 1) and then homogenized at 11 days old in 100 µl of a pH 7.4 M HEPES, 0.05M EDTA, 10% glycerol buffer. Tubes were centrifuged for a minute at 10,000 rpm and supernatant collected. Supernatant was further used for protein estimation, cholesterol and TAG measurements. Protein concentrations were determined

Figure 4.1: Timeline for chemical exposures, starvation, and polar lipid determinations in D. magna as described in Materials and Methods.
spectrophotometrically at 595 nm using Bradford Reagent (Bio-Rad, Hercules, CA, USA). Cholesterol concentrations were measured with a commercially available fluorometric assay kit (Cayman Chemical Co., Ann Arbor, MI) at an excitation wavelength of 550 nm and an emission wavelength of 590 nm (Amundson and Zhou, 1999) and the remaining supernatant was used to measure TAG concentrations with a commercially available colorimetric assay kit (Cayman Chemical Co., Ann Arbor, MI) at 540 nm (Fossati and Prencipe, 1982). All data was normalized to protein concentrations (µmoles cholesterol per mg protein or mg triacylglyceride per mg protein). Changes in protein, cholesterol and TAG concentrations were determined by one-way ANOVA followed by Fisher’s LSD as the post-hoc test in GraphPad Prism 6 (GraphPad Software, San Diego, CA) with a p-value < 0.01.

4.3.5 Lipid extraction and lipidomic analysis of adult and neonatal daphnids

We exposed 7-day old *D. magna* to DHA, LA, atrazine or triclosan for four days, and on day 11 adult and released neonates were euthanized. Embryos in the brood chamber were separated from the adults to ensure that lipids from these embryos or soon to be released neonates were not included in the lipid profiles. Polar lipids were extracted from the adult and neonatal daphnids separately with 5 tubes of 3 adult daphnids per treatment group and 30-60 neonates per tube produced from the broods of the 3 extracted adults. Lipid extractions were performed as described previously (Sengupta et al., 2016). Lipid profiles were determined by mass spectrometry (electrospray ionization triple quadrupole mass spectrometer from Applied Biosystems API 4000) analysis at the
Kansas Lipidomics Research Center (KLRC) (Isaac et al., 2007; Sengupta et al., 2016). Changes in overall lipid profiles were determined by two-way ANOVA with repeated measures followed by the Fisher’s LSD as the post-hoc test. PCA was performed with SAS 9.3 (SAS Institute Inc., Cary, NC) and hierarchical clustering performed with MultiExperiment Viewer (MeV) (Saeed et al., 2003) to confirm associations between specific lipids and chemical treatments.

4.3.6 RNA extraction and qPCR

Seven-day old daphnids were UT or exposed to DHA, LA, PA, atrazine or triclosan for 24-hours and 96-hours with 5 and 3 daphnids per beaker for 8- and 11-day old daphnids, respectively (n=5 beakers). RNA was extracted with the RNAeasy mini kit (Qiagen, Germantown, MD) and quantified with a spectrophotometer at 260/280 nm. cDNA was synthesized with MMLV reverse transcriptase. qPCR was performed as described previously with D. magna using 0.25X SYBR Green (Qiagen, Germantown, MD USA) and the iCycler from Bio-Rad Laboratories (Hercules, CA USA) to quantify expression (Ginjupalli et al., 2015; Sengupta et al., 2015). Genes quantified were HR96, forward primer 5’-TCT-GCG-AGG-CTT-TAG-GTT-3’ and reverse primer 5’-AGG-GCA-TTC-CGT-CTA-AAG-AAG-GCT-3’ at an annealing temperature of 58°C, magro – gastric lipase (CG5932), forward primer 5’-GCA-TAG-GAC-GTG-AGA-TGG-TTA-G-3’ and reverse primer 5’-ACA-AGA-AGC-TCG-CAT-GGT-TA-3’ at an annealing temperature of 51°C, mannosidase (CG9468) forward primer 5’-GGT-TCC-CTG-GAG-TTT-ATG-GTA-G-3’ and reverse primer 5’-AGT-CGT-CGG-TGA-ATC-
TGT-TG-3’ at an annealing temperature of 53°C, NPC1b (acid sphingomyelinase) forward primer 5’-TCA-TAG-GTG-GAC-AGC-AAG-ATT-AC-3’ and reverse primer 5’TAG-CAG-GCA-CAC-CAA-CAT-AG-3’ at an annealing temperature of 55°C, and Cer2 (ceramidase) forward primer 5’-GTG-CCT-TGT-GTA-AAG-TCG-AAA-C-3’ and reverse primer 5’-GGC-CAA-CCA-CTG-TGA-AAT-TAT-G-3’ at an annealing temperature of 60°C. qPCR results were normalized to the expression of β-actin (forward primer 5’-CCA-CAC-TGT-CCC-CAT-TTA-TGA-AG-3’ and reverse primer 5’-CGC-GAC-CAG-CCA-AAT-CC-3’) as the housekeeping gene as described previously (Heckmann et al., 2006) at an annealing temperature of 52.2°C. Statistical differences in gene expression were determined by one-way ANOVA followed by Fisher’s LSD with a p-value ≤ 0.05 (n = 5).

4.3.7 Acute and chronic toxicity of carmofur and GW4869

Forty-eight hour acute toxicity assays were performed with GW4869, a neutral sphingomyelinase inhibitor (Luberto et al., 2002) and carmofur, an acid ceramidase inhibitor (Pizzirani et al., 2013). GW4869 and carmofur were reconstituted in 99.7% DMSO (Fisher Scientific, Fair Lawn, NJ USA), and therefore DMSO was provided in equal concentrations in all replicates including controls (UT; untreated group) at 0.01%. Acute toxicity tests were performed with the chemicals using the following concentrations for each: 0.01, 0.1, 1, 10, 100 and 1000 µg/L. Less than 24-hour old daphnids were used in tests with four daphnids per treatment beaker and 5 beakers per
exposure group (USEPA, 2002). LC50 values and confidence intervals were determined from sigmoidal dose response curves generated by GraphPad Prism 6.

Reproductive toxicity caused by GW4869 and carmofur was determined by exposing <24-h *D. magna* neonates to these compounds in a 21-day standard chronic toxicity test ((ASTM), 1988). Daphnids were exposed to 10, 20, 50, 100, or 200 µg/L (0.039, 0.078, 0.19, 0.39 or 0.78 µM respectively) carmofur or 20, 50, 100, 200 or 400 µg/L (0.035, 0.09, 0.17, 0.35 or 0.69 µM respectively) GW4869 in 40 ml of culture medium for 21 days with renewals every other day. Daphnids were fed 3x10^6 *R. subcapitata* cells supplemented with 50 µl of an aqueous suspension of blended TetraFin fish flakes at 2.5 mg/ml dry weight once a day for first seven days and twice a day for remaining fourteen days as described previously (Sengupta et al., 2016). Statistical differences in survival was determined using Fisher’s exact test and reproduction was determined by one-way ANOVA with GraphPad Prism 6 (GraphPad Software, San Diego, CA) with Fisher’s LSD used as the post-hoc test (p ≤ 0.001).
4.4 Results

4.4.1 Neonates preferentially sequester sphingomyelin

Our previous work investigated lipid profiles in adolescents, how lipids are perturbed by diet or toxicants, and whether particular lipids are associated with starvation survival or reproduction (Sengupta et al., 2016). This study in part compares lipid profiles in adults and neonates and whether specific dietary lipids and toxicants preferentially alter lipid profiles in adults or neonates. Therefore, our first course of action was to determine if there are differences in lipid profiles between untreated adults and neonates. Interestingly, the polar lipidome of neonates contains a significantly higher percentage of sphingomyelin (SM) than adult daphnids at the expense of phosphatidylcholine (PC) (Figure 4.2). PC formed 79.8% of the *D. magna* lipidome in adults, which is similar to adolescents (75%) (Sengupta et al., 2016), while SM formed a small percentage of the lipidome (2.7%). However among neonates, PC only formed 56.4% of the lipidome and SM formed 21.8% of the lipidome, an 8.1X increase over adults.
Figure 4.2: Relative concentrations of different classes of polar lipids in untreated adult and neonatal daphnids. Data are analyzed by two-way ANOVA followed by Fisher’s LSD as the post-hoc test (n=5) (*p<0.0001). Phosphatidylcholine (PC), phosphatidylserine (PS), phosphatidylinositol (PI), phosphatidylglycerol (PG), phosphatidylethanolamine (PE), phosphatidic acid (PA), sphingomyelin (SM).

4.4.2 Starvation survival and fecundity in adult *D. magna*

Previous research demonstrated that dietary lipids and toxicants can alter starvation survival and fecundity in adolescent *D. magna*. For example, pre-treatment with triclosan repressed maturation and completely abolished reproduction in adolescent daphnids under starvation conditions (Sengupta et al., 2016). Therefore, we wanted to determine if specific lipids are associated with development, reproduction, or survival and in turn examine the compromises and lipid exchanges that occur between reproductive adults and their broods. Unexpectedly, exposure of adult *D. magna* to different lipids or toxicants did not significantly perturb starvation survival (Figure 4.3A).
All of the daphnids in each group died between 18-20 days-old or 7-9 days after starvation was initiated. It may be that adult daphnids do not have to continue the maturation process or they already allocated enough lipid resources from their normal diet and in turn each group demonstrated similar survival irrespective of the chemical exposures performed during days 7-10 prior to starvation.

However, this does not explain why exposed groups differ in fecundity. Reproduction under starvation condition is grouped under three tiers; A, D and F with A higher than UT and D and F lower than UT (Figure 4.3B). DHA (2 and 4 µM) and PA (4 µM) treatment groups demonstrate higher reproduction than the UT group. Triclosan (0.1 and 0.25 µM), PA (2 µM) and LA (4 µM) show lower reproduction than the UT group and atrazine (20 and 40 µM) and LA (8 µM) show the lowest reproduction among all treatment groups. Atrazine is the only chemical that reduces reproduction at the concentrations tested under normal, non-starvation conditions. The other chemicals tested do not repress reproduction at the concentrations tested under ideal feeding conditions in a typical chronic toxicity test (Sengupta et al., 2016). Interestingly, while not statistically significant, higher reproduction was weakly associated with shorter lifespan as the DHA and PA groups that showed the highest fecundity also died at the youngest age (at 18 days-old).

Neonates collected on day 16 from each of the treated, starved adults were followed for starvation-survival for fourteen days. None of the lipid treatments or the atrazine treatment extended the lifespan of their starved neonates (Figure 4.3C). Instead all fatty acid exposures (DHA, LA and PA) reduced starvation survival of the offsprings.
In contrast, maternal triclosan exposure significantly increased neonatal survival time. The daphnids did not develop probably due to the lack of food, but were able to survive in a senescent-like state. Similarly, in our previous study triclosan-exposed starved adolescent daphnids did not develop nor reproduce but were able to survive in a senescent-like state while other treatment groups continued to develop and reproduce (Sengupta et al., 2016). Thus, the combination of starvation and triclosan is initiating senescence. DHA and triclosan are both HR96 inhibitors, but each demonstrated different yet significant effects in this study under starvation conditions; with DHA leading to better reproduction among adult daphnids and maternal triclosan exposure leading to better neonatal survival (Figure 4.3).
Figure 4.3: Adult starvation survival (A), reproduction (B) and neonate starvation survival (C) in daphnids exposed to DHA, LA, PA, atrazine or triclosan. Data are presented as number of daphnids alive per day (A and C) or number of neonates per adult daphnid (B). Survival of daphnids was analyzed using Fishers 2x2 test (n=12), * represents differences from DHA, LA, PA and atrazine groups (p<0.05). Reproduction (B) was analyzed using one-way ANOVA with a general linear model followed by Fisher’s LSD (n=12) (p<0.05), with higher reproduction observed in group A than UT daphnids, and lower reproduction in groups D and F compared to UT and group A treated daphnids (n=12).
4.4.3 Cholesterol and TAG levels in adult daphnids pre-exposed to chemicals

HR96 is a regulator of magro (homolog to mammalian gastric lipase), which plays a key role in clearance of cholesterol and digestion and absorption of triacylglycerols in *Drosophila* (Sieber and Thummel, 2012). Each of the chemicals daphnids are exposed to in this study alter HR96 activity in vitro (Karimullina et al., 2012). We tested whether pre-exposure to HR96 activators (atrazine and LA) would cause lower cholesterol and higher TAG levels (Sieber and Thummel, 2012) relative to protein concentrations (not altered; Figure 4.4A), and tested whether HR96 inhibitors (DHA and triclosan) would cause the opposite in 11-day old *D. magna* (Figure 4.4). Our results show that atrazine, an HR96 activator reduced cholesterol as expected; however, so did triclosan (Figure 4.4B), an HR96 inhibitor (Karimullina et al., 2012). In addition, atrazine, but not the other treatments, unexpectedly reduced TAG (Figure 4.4C), which may in part be due to 3X higher protein levels. Therefore, the effect on cholesterol and TAG does not follow the expected pattern for HR96 modulators/inhibitors as cholesterol is not increased by inhibitors such as triclosan, and TAG is not increased by activators such as atrazine. Furthermore, none of the fatty acids perturbed cholesterol or TAG.
Figure 4.4: Protein, Cholesterol and TAG concentrations in seven-day old *D. magna* following four days of exposure to different chemicals. (A) Protein (µg/ml) concentrations (B) Cholesterol concentrations (µmoles cholesterol/mg protein) and (C) TAG levels (mg TAG/mg protein) determined in homogenized adult daphnids. Data are analyzed using one-way ANOVA followed by Fisher’s LSD test (n=5) and presented as mean ± SEM. An ‘a’ is different from UT, ‘b’ from DHA, no asterisk indicates a p<0.01 and an asterisk indicates a p<0.001.
4.4.4 Fatty acids and toxicants decrease phosphatidylcholine and increase sphingomyelin levels in daphnids

We hypothesized that dietary lipids and toxicants perturb the normal exchange of specific lipid species between reproductive adults and their broods. In addition, we examined whether there are specific fatty acids associated with the allocation of lipids towards starvation survival or reproduction. Of special interest are the PC and SM because their relative concentrations are different in adults than neonates. Triclosan-exposed adult daphnids, which showed reduced reproduction, bioconcentrate the most overall polar lipid content primarily through increased PC and PE (Table 4.1). Triclosan and atrazine-exposed daphnids also show poor lipid accumulation in their broods (Table 4.2) and this is associated with poor reproduction. Fatty acid treated daphnids (LA, DHA) surprisingly retained the least amount of polar lipids in the adults (Table 4.1); however, some of these lipids are allocated to the neonates as the LA-treated adults produced neonates with the highest levels of total polar lipids and PCs (Table 4.2) even though the LA-treated adults did not reproduce well indicating an increase in lipids/daphnid.
Table 4.1: Quantity of each lipid group found in polar lipids extracted from treated and untreated adult *D. magna*.

<table>
<thead>
<tr>
<th>Lipid</th>
<th>UT±</th>
<th>DHA (4µM)</th>
<th>LA (4µM)</th>
<th>Atr (40 µM)</th>
<th>Tric (0.25µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysoPC</td>
<td>0.042±0.018</td>
<td>0.031±0.008</td>
<td>0.030±0.044</td>
<td>0.031±0.013</td>
<td>0.031±0.017</td>
</tr>
<tr>
<td>PC</td>
<td>4.210±0.427</td>
<td>3.558±1.396</td>
<td>2.853±1.324</td>
<td>3.811±0.694</td>
<td>4.635±1.359</td>
</tr>
<tr>
<td>SM</td>
<td>0.145±0.028</td>
<td>0.117±0.028</td>
<td>0.104±0.061</td>
<td>0.120±0.039</td>
<td>0.166±0.043</td>
</tr>
<tr>
<td>LysoPE</td>
<td>0.038±0.014</td>
<td>0.065±0.023</td>
<td>0.029±0.012</td>
<td>0.058±0.019</td>
<td>0.065±0.018</td>
</tr>
<tr>
<td>PE</td>
<td>0.635±0.155</td>
<td>0.864±0.278</td>
<td>0.567±0.303</td>
<td>1.028±0.249</td>
<td>1.306±0.367</td>
</tr>
<tr>
<td>PG</td>
<td>0.070±0.019</td>
<td>0.028±0.022</td>
<td>0.050±0.039</td>
<td>0.030±0.009</td>
<td>0.039±0.014</td>
</tr>
<tr>
<td>PI</td>
<td>0.103±0.044</td>
<td>0.109±0.032</td>
<td>0.05±0.031</td>
<td>0.101±0.017</td>
<td>0.087±0.056</td>
</tr>
<tr>
<td>PS</td>
<td>0.034±0.010</td>
<td>0.037±0.012</td>
<td>0.029±0.019</td>
<td>0.038±0.007</td>
<td>0.028±0.010</td>
</tr>
<tr>
<td>PA</td>
<td>0.008±0.002</td>
<td>0.009±0.001</td>
<td>0.009±0.006</td>
<td>0.011±0.003</td>
<td>0.012±0.002</td>
</tr>
<tr>
<td>Lipids</td>
<td>5.286±0.619</td>
<td>4.818±1.715</td>
<td>3.721±1.584</td>
<td>5.227±0.610</td>
<td>6.368±1.816</td>
</tr>
</tbody>
</table>

£Data presented as mean nmol lipid/daphnid +/- SEM. Statistical differences determined by two-way ANOVA followed by Fisher’s LSD as the post-hoc test (p<0.05, *p<0.01, **p<0.001) (n=5).

‘a’ treatment different than UT
‘b’ treatment different than DHA
‘c’ treatment different than LA
‘d’ treatment different than Atr
‘e’ treatment different than Tric
Table 4.2: Quantity of each lipid type found in the polar lipids extracted from treated and untreated neonatal *D. magna* (per brood)

<table>
<thead>
<tr>
<th>Lipid</th>
<th>UT (4µM)</th>
<th>DHA (4µM)</th>
<th>LA (40 µM)</th>
<th>Atr (40 µM)</th>
<th>Tric (0.25µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysoPC</td>
<td>0.053±0.052</td>
<td>0.012±0.013</td>
<td>0.013±0.008</td>
<td>0.010±0.010</td>
<td>0.018±0.027</td>
</tr>
<tr>
<td>PC</td>
<td>0.573±0.370</td>
<td>0.277±0.175</td>
<td>0.791±0.642</td>
<td>0.384±0.437</td>
<td>0.323±0.409</td>
</tr>
<tr>
<td>SM</td>
<td>0.230±0.176</td>
<td>0.287±0.256</td>
<td>0.140±0.054</td>
<td>0.127±0.065</td>
<td>0.248±0.113</td>
</tr>
<tr>
<td>LysoPE</td>
<td>0.039±0.025</td>
<td>0.043±0.030</td>
<td>0.033±0.009</td>
<td>0.025±0.016</td>
<td>0.027±0.028</td>
</tr>
<tr>
<td>PE</td>
<td>0.087±0.051</td>
<td>0.090±0.057</td>
<td>0.090±0.019</td>
<td>0.061±0.043</td>
<td>0.078±0.099</td>
</tr>
<tr>
<td>PG</td>
<td>0.008±0.005</td>
<td>0.017±0.027</td>
<td>0.023±0.007</td>
<td>0.003±0.002</td>
<td>0.012±0.006</td>
</tr>
<tr>
<td>PI</td>
<td>0.012±0.009</td>
<td>0.005±0.004</td>
<td>0.014±0.010</td>
<td>0.006±0.006</td>
<td>0.011±0.017</td>
</tr>
<tr>
<td>PS</td>
<td>0.017±0.014</td>
<td>0.006±0.006</td>
<td>0.010±0.004</td>
<td>0.006±0.004</td>
<td>0.004±0.004</td>
</tr>
<tr>
<td>PA</td>
<td>0.006±0.002</td>
<td>0.009±0.006</td>
<td>0.007±0.004</td>
<td>0.007±0.001</td>
<td>0.008±0.004</td>
</tr>
<tr>
<td>Lipids</td>
<td>1.025±0.636</td>
<td>0.747±0.465</td>
<td>1.122±0.687</td>
<td>0.629±0.526</td>
<td>0.728±0.684</td>
</tr>
<tr>
<td>Neonate#</td>
<td>34.2±6.3797</td>
<td>47.4±10.7842</td>
<td>42±11.2916</td>
<td>52.6±13.6492</td>
<td>32.6±5.8566</td>
</tr>
</tbody>
</table>

£Data presented as mean nmol lipid/brood +/- SEM. Statistical differences determined by two-way ANOVA followed by Fisher’s LSD as the post-hoc test (p<0.05,*p<0.01,**p<0.001) (n=5).
Neonate#. Mean ± SEM number of neonates produced per daphnid.
‘a’ treatment different than UT
‘b’ treatment different than DHA
‘c’ treatment different than LA
‘d’ treatment different than Atr
‘e’ treatment different than Tric
Relative levels (percent total signal) of each lipid type were also examined given the differences in total lipids between treatments, and between adults and their broods (Figure 4.5). Different phospholipids are made from other types of phospholipids. For example, PEs can be methylated to make PCs, and SMs are often made from PEs or PCs. Therefore, the concentrations of these phospholipids may interact and the relative contributions to the total lipid pool may change (Figure 4.5). Both toxicants and fatty acids significantly lowered PC levels in comparison to the UT adult daphnids (Figure 4.5A). PC levels often went up in exposed adolescents (Sengupta et al., 2016). Adult PE levels were significantly lower in UT and LA groups in comparison to the DHA, atrazine and triclosan groups. In contrast, the broods showed increases in relative SM levels when exposed to DHA or triclosan, primarily at the expense of PCs (Figure 4.5B). This is an interesting result given that SMs are associated with neonates, DHA and triclosan are both HR96 inhibitors, DHA and triclosan both alter reproduction (although in opposing directions), and triclosan causes delayed maturity or senescent-like state.
Figure 4.5: Relative levels of different polar lipid classes in adult and neonatal daphnids following four days of chemical exposures. Changes in percent polar lipids were measured after exposure of adult (A) and neonatal (B) daphnids to different chemicals. Data are analyzed by two-way ANOVA followed by Fisher’s LSD as the post-hoc test (n=5). ‘An a’ is different from UT, ‘b’ from DHA, ‘c’ from LA, ‘d’ from atrazine (Atr), ‘e’ from triclosan (Tric). No asterisk indicates a p<0.01 and an asterisk indicates a p<0.001.
Therefore, we investigated if there are specific polar lipid species altered by chemical treatment or life stage. The Hierarchical Clustering using MeV confirmed that most of the individual PCs are reduced in adults following treatments in comparison to UT (Appendix C-2A). Among PE species the ones with 34-38 carbons were increased in adults; whereas PE 37:3, 37:4, 28:1 and 33:0 were decreased in adults. Only two SM species were altered, 32:2, which is produced a very low concentrations and 42:1, which is increased in several treatment groups. Principal Component Analysis (PCA) of the adult data sets revealed that DHA, atrazine and triclosan alter adult lipidomic signature in a similar fashion, and in an opposing fashion to daphnids exposed to LA and UT (Appendix C-2B).

Analysis of specific polar lipid species in neonates revealed that most of the PC species (low and mid-weight) are highly expressed in the LA group and moderately expressed in the other groups, with some mid-weight PCs (35:5, 37:5, 39:5 and 39:6) showing slightly increased production in the DHA, atrazine and triclosan exposed groups (Figure 4.6A). Among SMs the ones with a one double bond (32:1, 34:1, 36:1 40:1 and 42:1) were highly expressed in all groups with the exception of SM 36:1 and 42:1 that had low production in the LA group. PCA of the neonatal data sets revealed that DHA, atrazine and triclosan alter lipidomic signatures in a similar fashion, and are opposite in position from LA and to a lesser extent UT (Figure 4.6B) as also observed in adults (Appendix C-2B). Neonates released from LA exposed daphnids had higher levels of PC and total lipids compared to other groups (Table 4.2) and several low and mid-weight PC species were associated with LA in the PCA bi-plot (Figure 4.6B). Therefore, PC’s
appear to be associated with lower reproductive outcome potentially because of an increase in n-6 fatty acids that are not as crucial to health as the n-3 fatty acids. Overall, perturbations in specific PCs, PEs, and SMs in the neonates are common.
Figure 4.6: Significant changes in relative polar lipid species among neonatal daphnids. (A) Hierarchal Clustering (HCL) was used to cluster and visualize significant changes using MultiExperiment Viewer (MeV). One-way ANOVA (p<0.05) was used to identify significantly altered lipid species. (B) Principle component analysis (PCA) demonstrates associations between chemical exposures and lipid profiles in neonatal *D. magna*. 
4.4.5 Toxicant and lipid-induced changes in sphingomyelin concentrations in adults and neonates

SMs are of particular interest because they are predominantly found in neonates (Figure 4.2) and neonate concentrations are perturbed by DHA and triclosan (Figure 4.5). Several of the mid-weight SM species (i.e. 36:0, 36:1, 38:1 and 38:2) are neonate specific (Figure 4.7). Total polar lipid concentrations among neonates reveal that DHA caused significant alterations in SM species 36:1 and 36:2 in comparison to the toxicants, and triclosan caused significant alteration in 36:0 in comparison to untreated and LA groups (Figure 4.7). Among adults, concentrations of specific SM species such as 32:2, 34:2, 40:0, 42:1 and 42:2 were significantly altered in the control group in comparison to the treatment groups (DHA, LA, atrazine and triclosan). In summary, several mid-weight SMs are neonate specific and these and other SMs are altered in opposing directions by LA and DHA with triclosan showing a similar pattern to DHA.
Figure 4.7: Changes in sphingomyelin (SM) composition among different exposure groups in adult and neonatal daphnids. Changes in concentrations (nmol) of individual SM species following chemical exposure (UT, DHA, LA, atrazine and triclosan) in adult and neonatal daphnids were analyzed by two-way ANOVA followed by Fisher’s LSD (n=5) (p<0.05). An ‘a’ refers to UT different from DHA, ‘b’ refers to UT different from LA, ‘c’ refers to UT different from Atr, ‘d’ refers to UT different from Tric, ‘e’ refers to DHA different from LA, ‘f’ refers to DHA different from Atr, ‘g’ refers to DHA different from Tric, ‘h’ refers to Tric different from LA, and ‘i’ refers to Tric different from Atr.
4.4.6 Perturbations in the regulation of genes involved in lipid absorption and sphingomyelin metabolism

HR96 regulates the absorption of triacylglycerols and the metabolism of sphingomyelins in Drosophila (King-Jones et al., 2006; Sieber and Thummel, 2009). Increased sphingomyelins are involved in the Niemann-Pick disease, a lysosomal disorder (Bujold et al., 2010). Therefore, we investigated the expression of these genes to determine if they are age-dependent or perturbed by chemical exposure. HR96 and magro are expressed equally at all age groups; however, mannosidase and NPC1b (sphingomyelinase) expression is higher in older daphnids. Ceramidase expression was lower in the 14-day old daphnids in comparison to 7-day old daphnids (Table 4.3). It is possible that the greater expression of NPC1b may be important in SM regulation, breakdown, use, and development during early reproduction.

Table 4.3: Age-dependent expression of genes in untreated *D. magna*

<table>
<thead>
<tr>
<th>Gene</th>
<th>2-day old£</th>
<th>4-day old</th>
<th>7-day old</th>
<th>14-day old</th>
</tr>
</thead>
<tbody>
<tr>
<td>HR96</td>
<td>1.00 ± 0.26</td>
<td>1.30 ± 0.17</td>
<td>1.95 ± 0.63</td>
<td>1.11 ± 0.09</td>
</tr>
<tr>
<td>magro</td>
<td>1.00 ± 0.64</td>
<td>0.65 ± 0.67</td>
<td>0.66 ± 0.67</td>
<td>0.84 ± 0.92</td>
</tr>
<tr>
<td>mannosidase</td>
<td>1.00 ± 0.69</td>
<td>0.55 ± 0.42</td>
<td>0.521±0.369</td>
<td>6.11 ± 4.34abc</td>
</tr>
<tr>
<td>NPC1b</td>
<td>1.00 ± 0.24</td>
<td>1.15 ± 0.45</td>
<td>1.801 ± 0.71a</td>
<td>2.52 ± 0.61ab</td>
</tr>
<tr>
<td>Cer2</td>
<td>1.00 ± 0.53</td>
<td>1.39 ± 0.46</td>
<td>1.505 ± 1.011</td>
<td>0.53 ± 0.29c</td>
</tr>
</tbody>
</table>

£Data presented as mean relative expression +/- SEM. Statistical differences determined by one-way ANOVA followed by Fisher’s LSD as the post-hoc test (p<0.05) (n=4-5). ‘a’ expression different than 2-day old, ‘b’ expression different than 4-day old, ‘c’ expression different than 7-day old
Seven-day old daphnids were treated for either 24 or 96 hours; until they were 8 or 11 days old. Expression of HR96 and HR96-regulated genes, magro, mannosidase, NPC1b (sphingomyelinase), and Cer2 (ceramidase) were quantified using qPCR (Figure 4.11). Based on the literature, we expected HR96 activators would induce expression of HR96, magro and sphingomyelinase, and reduce the expression of mannosidase (King-Jones et al., 2006; Sieber and Thummel, 2009). We thought HR96 inhibitors would act in an opposing fashion. The regulation of Cer2 by HR96 is not known, but we considered it likely that this enzyme important in sphingosine production may also be regulated through HR96. The saturated fatty acid, PA was used as a lipid control.

qPCR results demonstrate that all the lipids regulate the HR96-regulated genes after a 24 hour exposure (Figure 4.8). Magro is increased by PA and LA 45-85X, and down-regulated by DHA 5X. Down-regulation of magro by DHA is not significant by ANOVA when compared to PA/LA, but significant by ANOVA when compared to UT (p < 0.001). The magro data is directionally consistent with our expected results for LA and DHA. However, mannosidase and NPC1b are down-regulated by all the lipid treatments. This may indicate differential regulation of these genes by lipids or differences between *Drosophila* and *Daphnia*. After 24 hours there were few changes in the expression of the HR96 regulated genes following toxicant exposure with the exception of mannosidase, which is down-regulated by triclosan. Atrazine increased ceramidase and HR96 expression at 20 µM, but not in a concentration-dependent fashion (Figure 4.8).
Figure 4.8: Altered expression of HR96 (A), magro (B), mannosidase (C), NPC1b (D) and Cer2 (E) in 8 day-old *D. magna* exposed to fatty acids or toxicants for 24 hours. Statistical significance was determined by one-way ANOVA followed by Fisher’s LSD with *p ≤ 0.05 considered significant. Data are provided as mean ± SEM (n = 5).
After 96 hours of exposure to the chemicals, the effects of the lipids were not as strong. In fact, magro was down-regulated by PA and LA. Mannosidase was also still down-regulated with intermittent induction of ceramidase (Figure 4.9). However, the toxicants now showed greater effects than after 24 hours of exposure. The potent HR96 activator, atrazine (Sengupta et al., 2015) increased HR96, NPC1b, and ceramidase expression and down-regulated mannosidase as originally hypothesized (Figure 4.9). However, magro was not regulated by atrazine. Triclosan’s effects were more mixed, suggesting that triclosan may not be a potent HR96 inhibitor in vitro or its inhibitory effects are not observed transcriptionally without an inducer present.
Figure 4.9: Altered expression of HR96 (A), magro (B), mannosidase (C), NPC1b (D) and Cer2 (E) in 11 day-old *D. magna* exposed to fatty acids or toxicants for 96 hours. Statistical significance was determined by one way ANOVA followed by Fisher’s LSD test with *p* ≤0.05 considered significant. Data are provided as mean ± SEM (n = 5).
4.4.7 Ceramidase inhibitor Carmofur alters fecundity in *D. magna*

Because sphingomyelins are associated with reproduction and development and there are changes in the expression of sphingomyelinase and ceramidase following chemical treatment, we investigated whether the sphingomyelinase inhibitor, GW4869 or the ceramidase inhibitor, carmofur repress fecundity. First acute toxicity tests were performed to determine concentrations for the chronic toxicity tests. GW4869 caused no death at concentrations up to 1 mg/L and carmofur did not induce death until 1 mg/L with an LC50 estimated at 859 µg/L.

GW4869 had no significant effects on reproduction (Figure 4.10). However, the ceramidase inhibitor carmofur reduced reproduction in a concentration-dependent manner due to a delay in maturation and reproduction (Figure 4.10). Instead of initial reproduction occurring on day 9 as in controls, it occurred on day 14 in daphnids exposed to 100 µg/L and day 16 in daphnids exposed to 200 µg/L carmofur (0.78 µM). Overall, reproduction was reduced by approximately 70% in daphnids exposed to 200 µg/L carmofur. This data suggests that the production of sphingosine from SMs is important in reproduction.
Figure 4.10: Altered fecundity of adult daphnids exposed to carmofur (A) or GW4869 (B). Statistical significance was determined by one-way ANOVA followed by Fisher’s LSD with *p<0.001 considered significant (n=10).
4.5 Discussion

Sphingomyelin production is age specific. The percentage of SMs in polar lipids in neonatal daphnids is 8.1X higher than in adults (Figure 4.2). Several SMs are specific to neonates, including 36:0, 36:1, 38:1 and 38:2 (Figure 4.7). This suggests a key role of several SM species in maturation and development. Therefore, we hypothesized that inhibition of the breakdown of SMs into ceramides or sphingosine would severely impact maturation and fecundity, and this proved true (Figure 4.10).

SMs are key polar lipids involved with cell signaling, growth and development (Cutler et al., 2014). SMs are converted to ceramide by sphingomyelinases, and ceramides are converted to sphingosine (involved in reproduction) by ceramidases (Hannun and Obeid, 2008; Cutler et al., 2014). HR96 regulates several genes in the uptake of lipids and the metabolism of SM (Figure 4.11), including magro and NPC1b, a sphingomyelinase (Sieber and Thummel, 2009; Bujold et al., 2010; Sieber and Thummel, 2012). Increased sphingomyelinase activity would increase ceramide and ceramidase would increase sphingosine (Hannun and Obeid, 2008). Ceramides, SMs, sphingosine and sphingosine-1-phosphate are involved in reproduction and development in Drosophila (Herr et al., 2003; Phan et al., 2007), C. elegans (Cutler et al., 2014), and mice (Jee et al., 2011). Thus we predict that HR96 modulators would alter the expression of genes involved in the uptake and metabolism of sphingomyelins and in turn activators would reduce SM concentrations and inhibitors would increase SM concentrations by inhibiting SM metabolism.
Figure 4.11: Sphingomyelin metabolism pathway. Genes regulated by HR96 play important roles in breakdown of sphingomyelin to ceramide and sphingosine.

We tested four HR96 regulators and found that SMs were significantly increased by the HR96 inhibitors, DHA and triclosan. DHA also altered gene expression of key HR96-regulated genes, including repressing sphingomyelinase expression consistent with a decrease in SM metabolism and increased retention. However, DHA exposure was not associated with a decrease in metabolism, but instead an increase in metabolism. The mechanism is unknown, but we think that DHA is providing the proper dietary lipids for
the production of ceramides and sphingosine while also reducing SM metabolism so that it can be retained or used for other purposes.

Considering that the lipidomics data also indicated that triclosan increased SM levels in neonates, (Figure 4.5B), we expected to see down-regulation of magro and sphingomyelinase by triclosan similar to DHA. We did not observe down-regulation with triclosan. Therefore, we predict that triclosan is increasing SM in neonates by perturbing a different pathway as triclosan did not alter the expression of HR96-regulated genes. Triclosan is a potential fatty acid synthase (FASN) inhibitor (Sadowski et al., 2014), and in turn could decrease the accumulation and use of lipids. Furthermore, triclosan may directly inhibit sphingomyelinase, ceramidase, or another key lipid involved in the metabolism or production of SM. This effect may not manifest itself under ideal conditions, but only during diet restriction because of competition from other lipid sources. Triclosan’s effects on development in adolescents (Sengupta et al., 2016), and neonates are very interesting and could have significant adverse effects on the environment because of a lack of maturation and reproduction. However, triclosan’s mechanism, which should be an interesting study in future research, does not appear to be regulated through HR96.

In contrast, atrazine induces the expression of HR96, sphingomyelinase and ceramidase in 11-day old D. magna (Figure 4.9), consistent with HR96 activation (Sieber and Thummel, 2009; Karimullina et al., 2012). Atrazine provides protection from the HR96 inhibitors, DHA and triclosan, potentially through HR96 activation that leads to increased glutathione S-transferase activity (Sengupta et al., 2015). However, atrazine has
no effect on magro, a key biomarker of HR96 activation (Sieber and Thummel, 2012). LA induces the expression of magro and represses mannosidase and NPC1b expression all key genes in the HR96 pathway. LA and atrazine do not increase SM concentrations and LA-treated neonatal daphnids have the lowest SM levels. LA exposure is also associated with PC (Figure 4.6), not SM and both LA and atrazine exposed daphnids show poor fecundity.

Given that the metabolites of SMs such as ceramides and sphingosine control several developmental processes in animals (Cutler et al., 2014); we hypothesized that the increase in SMs may be caused by reduced production of ceramide and sphingosine, and this in turn could lead to poor development. It is unknown if blocking the production of these enzymatic pathways has physiological consequences in *D. magna*. Carmofur, a ceramidase inhibitor that regulates the production of sphingosine severely reduced reproduction primarily by delaying development (Figure 4.10). However, GW4869, a sphingomyelinase inhibitor that blocks production of ceramides did not impact fecundity (Figure 4.10). This further supports the notion that the production of sphingosine (or sphingosine-1-phosphate) (Hannun and Obeid, 2008) is key in development and reproduction. This would also indicate that the ceramides are not important in reproduction and development. However, it is also possible that this particular sphingomyelinase inhibitor (GW4869) is unable to target the *Daphnia* specific sphingomyelinase and therefore ceramides are still produced and involved in the reproductive pathway. Accumulation of ceramides has been known to play a role in age-related apoptosis in mice germ cells (Perez et al., 2005). Ability of ceramides to induce a
senescent phenotype has also been demonstrated in a cell-culture model (Venable et al., 1995), and this could also in part describe why triclosan and DHA have very different phenotypes.

Low food resources such as diet restriction plays a positive role in increasing lifespan of certain species but not all species (Mair and Dillin, 2008; Latta IV et al., 2011). During food shortage organisms can allocate resources towards survival and somatic maintenance until normal conditions are available for development or reproduction. Previous work demonstrated that adolescent daphnids pre-exposed to triclosan and then starved did not develop into adulthood and entered a state of senescence (Sengupta et al., 2016). In this study, we demonstrate that adults exposed to triclosan and then starved released neonates that are resistant to starvation possibly because of their ability to enter senescence (Figure 4.3C). The reason for this is unknown; however, it is interesting to speculate that at least in part the inability to produce a significant amount of SM metabolites such as ceramides and sphingosine may play a role. In our current study maternal exposure to triclosan led to moderate reproduction, lower than the control group (Figure 4.3B) and interestingly higher survival among neonates born from these triclosan exposed adults (Figure 4.3C). This indicates that triclosan leads to a resource allocation towards survival and away from reproduction in young daphnids in both the studies. The survival is clearly a benefit to the population; however, the loss of development may severely impact the population if food resources do not become available.
Interestingly, dietary lipids such as DHA also increased the levels of SMs similar to triclosan. Overall DHA had very similar lipid profiles to the toxicants atrazine and triclosan and opposite profile to LA in this study for both neonates (Figure 4.6) and adults (Appendix C-2). DHA exposure demonstrated better reproduction in this study (Figure 4.3) and it was also associated with better reproduction in our previous study with adolescent daphnids (Sengupta et al., 2016). LA on the other hand was associated with poor reproduction, but the broods from LA-exposed adults had higher lipid levels and therefore may have been able to better deal with some stressors. However, to our surprise this did not include neonatal starvation survival. Overall, DHA is a key component of a healthy algal diet for *Daphnia* (Brett and Muller-Navarra, 1997). It is possible that a healthy diet may overcome some of the adverse effects mediated by toxicants.

Toxicants can alter energy utilization. Activating key xenobiotic sensing transcription factors such as CAR, PXR, and AhR in mammals alters energy utilization and is associated with alterations in lipid allocation (Ueda et al., 2002; Shindo et al., 2007; Dong et al., 2009; Hernandez et al., 2009; Lu et al., 2015). Therefore, activation of HR96 in daphnids could do the same. Interestingly, HR96 activators such as LA and atrazine reduced reproduction, while the HR96 inhibitor, DHA increased reproduction. We propose that some of the adverse effects observed are consistent with changes in SM metabolism as both neonatal SM levels and the expression of key HR96 regulated genes are altered in chemical and age-dependent mechanisms. Methods for determining ceramides and sphingosines in neonatal daphnids are underway, but are not trivial. Of
special interest was the senescent like behavior observed in triclosan pre-treated daphnids that are starved. However, changes in gene expression observed are not consistent with HR96 activation unlike that observed for atrazine, LA, and DHA.

In conclusion, it is interesting to speculate that a drop in sphingosine, a key developmental lipid, may be associated with greater longevity and slow or no development. In addition, fatty acids and toxicants alter energy homeostasis and probably in some cases through similar pathways. Thus, the toxicants can cause sublethal effects by altering the resource allocation and metabolism of signaling lipids and in turn perturb survival under less than ideal conditions while reducing reproduction, and repressing development.

4.6 References


Ginjupalli, G.K., Baldwin, W.S., 2013. The time- and age-dependent effects of the juvenile hormone analog pesticide, pyriproxyfen on Daphnia magna reproduction. Chemosphere 92, 1260-1266.


CHAPTER FIVE
LOBSTERS ARE NOT SENSITIVE TO JUVENILE HORMONE ANALOGS

5.1 Abstract

The American lobster (*Homarus americanus*), a commercially important species is potentially exposed to pesticides extensively used on the blueberry fields of Maine. Some of these pesticides belong to the class of juvenile hormone (JH) analogs capable of altering growth and development among a wide range of arthropod species. We investigated the effects of three JH analogs; pyriproxyfen, methoprene and fenoxycarb on the survival and development of larval lobsters (from stage 1 to 4) in the presence and absence of the polyunsaturated fatty acids (PUFAs) docosahexaenoic acid and arachidonic acid that contribute to crustacean development and reproduction. Our results indicate that PUFA supplementation did not contribute towards faster development or provide protection from the JH analogs. In addition, none of the JH analogs caused toxicity at the concentrations tested indicating that it is unlikely these pesticides will reach environmental concentrations necessary to cause toxic effects in larval American lobsters.

5.2 Introduction

The American lobster *Homarus americanus* is a commercially important species in the northeastern United States and Canada. According to the United State’s National Marine Fisheries Service’s 2008 data, the majority (78%) of lobsters are harvested in Maine (Cawthorn, 2011). Maine is also the largest producer of lowbush blueberry
(Vaccinium angustifolium) in North America, with the United States Department of Agriculture estimating the total production of blueberries in Maine to be 83 million pounds in 2010 (Choate and Drummond, 2013). Insecticides are commonly used in the blueberry fields to combat infestations from multiple insects. Pyrethroids and growth regulators such as methoprene are some of the most commonly used pesticides. Methoprene and other growth regulators that work through as juvenile hormone (JH) analogs are of concern (Mistler, 2014). These anthropogenic compounds form a part of agricultural runoffs and may run into coastal waters where marine organisms such as lobsters may be exposed to these compounds.

JH plays a significant role in insect development and metamorphosis (Riddiford, 2008; Jones et al., 2010). In crustaceans, methyl farnesoate (MF) an unepoxidated form of JH III acts as the mediator of JH-like functions (Laufer et al., 1987). MF regulates reproductive maturity (Ginjupalli and Baldwin, 2013) and environmental sex determination (male production) in cladocerans such as Daphnia, Moina and Ceriodaphnia species (Oda et al., 2005). Popular JH analog pesticides such as methoprene, pyriproxyfen and fenoxycarb have similar effects (Olmstead and LeBlanc, 2003; Ginjupalli and Baldwin, 2013). In decapod crustaceans, JH analogs have been shown to perturb larval development, egg maturation and reproduction (McKenney, 2005). Methoprene has been shown to cause lethality in stage 2 larval lobsters at concentrations as low as 1 - 10 µg/L (Walker et al., 2005). The effects of other JH analogs have not been evaluated and therefore these chemicals may prove to be better alternatives or similarly toxic. Therefore, we are interested in determining the adverse
effects of three JH analogs on the survival and development of larval lobsters from stage 1 to stage 4 with special emphasis on pyriproxyfen.

Polyunsaturated Fatty Acids (PUFAs) are crucial energetic lipids that also act as signaling molecules involved in growth, development, immunity and energy metabolism (Catala, 2013). Phytoplankton rich in PUFA contribute towards enhanced growth and reproduction in invertebrates and vertebrates (Brett and Muller-Navarra, 1997). Arachidonic acid (AA; n-6) and docosahexaenoic acid (DHA; n-3) are crucial lipids in arthropod physiology. *Daphnia* supplemented with DHA or AA demonstrate higher growth rates and fecundity (Brett and Muller-Navarra, 1997; Ginjupalli et al., 2015). In addition, AA provides protection from pyriproxyfen-induced male production in female colonies of the parthenogenetic *Daphnia magna* (Ginjupalli et al., 2015).

The purpose of our studies is to determine the adverse effects of three different JH analogs with special emphasis on pyriproxyfen as a potential substitute for methoprene because there are concerns methoprene is toxic to lobsters (Bircher and Ruber, 1988; Mistler, 2014). In addition, we are interested in determining if minimal AA or DHA could provide greater growth or faster maturity, similar protection from pyriproxyfen in *H.americanus*, as it does for *D. magna*. This could provide an application for PUFA supplementation of diet.

5.3 Materials and Methods

Stage 1 lobster larvae were obtained from the Mount Desert Oceanarium (South West Harbor, ME) the day of hatching and cultured at the Mount Desert Island
Biological Laboratory (MDIBL), Salsbury Cove, ME using fresh saltwater. Lobsters were maintained in 1 pint mason jars with 300 ml of saltwater per jar under constant aeration at a temperature of 19-20ºC and a light: dark cycle of 15:9 (Figure 5.1). Two-thirds of the saltwater media was changed every day and the larvae were fed 2ml of *Artemia salina* twice daily (Burridge et al., 1999; Pruell et al., 2000). Pyriproxifen, methoprene, fenoxycarb, DHA and AA (Sigma-Aldrich, St. Louis, MO USA) stock solutions were dissolved in absolute ethanol (Sigma-Aldrich Chemical Co., Inc, Milwaukee, WI USA).

**Figure 5.1:** Lobsters were cultured in natural Maine seawater in 1 pint mason jars under constant aeration as shown
The developmental effects of pyriproxyfen (125 and 500 ng/L; 0.388 and 1.55 nM, respectively), AA (2 μM), DHA (2 μM) and mixtures of pyriproxyfen with DHA or AA on < 24 hour larval lobsters through stage 4 were determined over 14 days (n = 5-6). We evaluated lobsters from stage 1 to stage 4 because these early stages and likely most sensitive to effects on molting and growth. Stages 1 through 3 larvae are zoeal stages that float near the surface, while stage 4 is the first post-larval stage. Stage 4 lobsters have undergone metamorphosis and settle and hide in the benthos until they are much larger and not as susceptible to predation (Ennis, 1995). Toxicity has been observed in shrimp and crabs undergoing metamorphosis (McKenney, 2005). Chemical concentrations used in the developmental and toxicity study were determined based on previously published LC50 values for D. magna (Ginjupalli and Baldwin, 2013; Ginjupalli et al., 2015; Sengupta et al., 2015). All treatments and controls contained 0.002% ethanol. Lobsters were individually cultured as described above. Survival and time to stage 4 was observed.

Survival in groups of 3-4 was monitored (n = 4-5) to determine survival and quantify aggressive behavior. Stage 1 lobsters were exposed to ethanol (0.001%), untreated no ethanol, no aeration, pyriproxyfen (125-1000 ng/L; 0.388-3.10 nM), fenoxycarb (2 μg/L; 6.64 nM), or methoprene (500 μg/L; 1.61 μM) dissolved in ethanol (0.002%). Lobsters were fed and media changed daily as described above. Survival was monitored daily. Differences in developmental toxicity and chronic toxicity among the exposure groups were analyzed using life-test procedure with SAS 9.3 (SAS Institute
Inc., Cary, NC) and graphs were generated using GraphPad Prism 6 (GraphPad Software, San Diego, CA).

5.4 Results

As we refined our methods we first investigated survival of lobster larvae with and without aeration. Aeration of stage one larvae is necessary. Five days after initiating the culture, survival of aerated larvae was 100%; survival of non-aerated larvae was 10% (data not shown). Therefore, all further studies were performed with aeration.

Larval lobsters were treated with pyriproxyfen (125 and 500 ng/L), 2 µM AA, 2 µM DHA, or mixtures of pyriproxyfen with either AA or DHA to determine the effects of these compounds on survival and development. However, neither survival nor development of the lobsters through stage 4 was perturbed by 125 or 500 ng/L pyriproxyfen (Figure 5.2). Furthermore, neither AA nor DHA significantly decreased survival or development time alone or in combination with pyriproxyfen. One larval lobster died in the control, DHA and pyriproxyfen groups during the study and all surviving lobsters developed into stage 4 juveniles between days 10-14 of the study. One animal in the DHA group developed as early as day 10 with most of the other larvae developing into stage 4 lobsters during days 13-14 (Figure 5.2). Therefore, neither the PUFAs nor pyriproxyfen significantly perturbed survival or development to stage 4 at the concentrations tested.
Figure 5.2: Survival (A) and development (B) of lobsters from stage 1 to 4 following exposures to DHA, AA, DHA + pyriproxyfen (PF) and AA + PF.

In contrast, pyriproxyfen repressed development to reproductive maturity in *D. magna* following exposure of juveniles to concentrations as low as 50 ng/L (155 pM) (Ginjupalli and Baldwin, 2013). Concentrations as low as 25 - 50 ng/L (78 - 155 pM) have also been shown to induce male production in daphnids (Olmstead and LeBlanc, 2003).
2003; Ginjupalli and Baldwin, 2013). In this study, concentrations 40X higher had no discernable effect on American lobsters. Therefore, pyriproxyfen is unlikely to cause environmentally relevant effects on juvenile lobsters at concentrations (125-1000 ng/L) used in the study.

We hypothesized that juvenile hormone analogs may be reducing activity and in turn aggressiveness. Therefore chemical exposure may increase survival in group housed lobsters as group housed lobsters often kill each other (Tamm and Cobb, 1978) and the lobsters with low activity would be less likely to inflict lethal wounds. In addition, to pyriproxyfen, we also examined methoprene and fenoxycarb’s effects on lobsters at concentrations above environmentally relevant concentrations. Survival among group cultured lobsters was not significantly affected by exposure to any of the JH analogs, pyriproxyfen, methoprene or fenoxycarb (Figure 5.3). This indicates that none of these juvenile hormone analogs have toxic effects on juvenile lobsters.
Figure 5.3: Survival of larval lobsters following exposure to pyriproxyfen (PF), fenoxycarb (FC) and methoprene (MP)

Cladocerans such as *Daphnia magna*, *Moina macrocopa*, *M. micrura*, *Ceriodaphnia dubia* and *C. reticulata* are more sensitive to much lower concentrations of JH analogs (Oda et al., 2005) than decapod crustaceans such as *Palaemonetes pugio* (grass shrimp) and *Rhithropanopeus harrisii* (mud crab) (McKenney, 2005). The EC50 for production of male neonates in cladocerans is in the range of 220 to 9300 ng/L for fenoxycarb. *D. magna* is the most sensitive (220 ng/L) and *C. reticulata* and *M. macrocopa* least sensitive (Tatarazako et al., 2003; Oda et al., 2005). Pyriproxyfen’s effects on environmental sex determination in cladocerans have only been examined in *D. magna*, but effects have been observed at concentrations as low as 25-50 ng/L (Ginjupalli and Baldwin, 2013). *Daphnia* are much less sensitive to methoprene as
male production did not occur until 310 µg/L; more than 1000X higher than the other JH analogs (Wang et al., 2005). Concentrations of these chemicals used in our study with American lobsters are 20 (pyriproxyfen), 9 (fenoxycarb) and 1.7 (methoprene) times greater than the effect concentrations for each of these compounds in Daphnia.

5.5 Discussion

Studies with the decapod crustaceans such as shrimp and crab indicate that decapods are insensitive to the effects of JH analogs and do not show toxicity until µg/L and sometimes nearly mg/L concentrations. For example, toxicity on metamorphosis does not occur in grass shrimp or mud crab following methoprene (1000 µg/L in shrimp, 100 µg/L in crab), fenoxycarb (45 µg/L in shrimp, 240 µg/L in crab) or pyriproxyfen (>100 µg/L in shrimp, 50 µg/L in crab) unless high µg/L concentrations are applied (McKenney, 2005). However, one study in American lobster indicates that stage 2 lobsters are very sensitive to methoprene as concentrations as low as 10 µg/L reduced survival 72% below untreated lobsters (Walker et al., 2005). Our studies found that American lobsters were not sensitive to these compounds at low µg/L concentrations. Our data are more in line with studies in crab and shrimp than the studies with larval lobsters.

Environmental concentrations of pyriproxyfen determined in the past have been in the range of 93-400 ng/L (Schaefer and Miura, 1990; Serafini, 2001). Environmental concentrations of methoprene, as reported in natural waters of USA are in the range of 0.39-8.8 µg/L (Knuth, 1989) and in the range of 0.1-0.6 µg/L in surface waters (Johnson
and Kinney, 2006). Fenoxycarb levels have been measured at around 400 ng/L in experimental ponds 24-48 hours after aerial application (Schaefer et al., 1987). All of these concentrations are well below the concentrations we tested indicating that these chemicals are unlikely to cause adverse effects on decapod species.

Although there is indication that compounds like methoprene and fenoxycarb possibly alter larval development through disruption of juvenoid signaling and not through interaction with ecdysone receptor (EcR) in American lobsters (Tarrant et al., 2011), this study did not observe physiological effects associated with reduced molting or growth. Furthermore, metamorphosis, which is controlled by JH signaling, was also not perturbed by JH analogs at the concentrations tested as observed by exposing lobsters through stage 4 when metamorphosis to a post-larval stage occurs. Thus, no effect was observed on these crucial, commercially important crustaceans at the larval stage and during metamorphosis; typically a sensitive stage for JH analogs (Meola et al., 2000; Wang et al., 2005).

However within the scope of this study we were not able to investigate the effects of JH analogs on all stages of lobster development; so it may still be possible JH analogs such as pyriproxyfen may perturb development or increase male production as observed in other crustaceans, but during other windows such as on embryos. It is possible that larval lobsters may not prove to be a sensitive stage to determine the effects (if any) of these chemicals on maturity and development.

In addition, the fate, transport and bioavailability of pesticides in estuarine environments are determined by their individual chemical structures, salinity and
dissolved organic matter in the water (Mézin and Hale, 2004). Some studies have suggested that salinity alone can increase toxicity and bioaccumulation of organic compounds in estuarine organism such as larval grass shrimp by increasing bioavailability through reduced solubility (Hamelink et al., 1994). The effect of salinity is also suggested to be species dependent; as individual organisms have different physiological responses to changes in internal osmotic volume (Weinstein, 2003), and therefore salinity may reduce the toxicity of chemicals to marine organisms through other means including reduced bioavailability.

In conclusion, even though juvenile hormone analogs are known to disrupt embryogenesis and interfere with metamorphosis by inhibiting end stage larval development in crustaceans (Olmstead and LeBlanc, 2003; McKenney, 2005), they did not demonstrate such effects in the American lobster at concentrations significantly greater than found in the environment. Therefore, we conclude that it is unlikely that pyriproxyfen or other juvenile hormone agonists reach the environmental concentrations necessary to cause toxic effects in American Lobster.

5.6 References


Ginjupalli, G.K., Baldwin, W.S., 2013. The time- and age-dependent effects of the juvenile hormone analog pesticide, pyriproxyfen on Daphnia magna reproduction. Chemosphere 92, 1260-1266.


HR96 – the ligand activated transcription factor in *D. magna* is both a xenobiotic-sensor and a lipid-sensor; henceforth it can regulate the metabolism of both toxicants and fats. The overall purpose of our research was to determine if chemical-chemical or chemical-diet interactions can occur and if they are regulated through the same pathway by the HR96 receptor. Some of our major findings from this research were associated with chemical-chemical interaction involving the herbicide atrazine and other chemicals (toxicant or fat) leading to induction of a protective response possibly through activating HR96. We also observed a chemical-diet interaction involving the anti-fungal agent triclosan and diet-restriction or starvation; leading to perturbation of lipid metabolism and slowing development in daphnids. The key lipid associated with development in *D. magna* was found to be sphingomyelins and we also confirmed its significantly higher levels in neonates in comparison to adolescent and adult daphnids. Specific lipid alterations, induced gene expressions and consistent phenotypes associated with some of these chemical and diet exposures in our study, revealed the connection between HR96 and the sphingomyelin metabolism pathway.

For our first aim we were able to determine that at supraphysiological concentrations, the HR96 activator atrazine (20-80 µM) decreases the toxicity of HR96 inhibitors triclosan and DHA, presumably by activating HR96 and inducing protective enzymes. We confirmed that atrazine pretreatment increased GST activity and induced production of antioxidative enzymes. We concluded that the increase in GST activity and
TROLOX equivalent antioxidant capacity consistent with atrazine’s activation of HR96 could provide protection from a wide range of toxicants that induce ROS, including triclosan and unsaturated fatty acids predisposed to lipid peroxidation. Because the HR96 activator atrazine activates detoxification responses in *D. magna* in the presence of HR96 inhibitors, it is worthwhile to pursue further research in this area to confirm if this is being regulated by the HR96 activation pathway. Further experiments including acute mixture toxicity tests can be performed to understand if atrazine is able to provide protection from the toxicity of other toxicants. Currently our lab is trying to determine atrazine’s global gene expression by RNA sequencing in collaboration with Clemson University Genomic Institute. This will give us some future directions as to which specific detoxification genes are being upregulated by atrazine.

Knowing that atrazine is able to induce some key detoxification enzymes in *D. magna* we are curious if this could have some other applications in the field of mechanistic toxicology. The concentrations at which atrazine provided protection (20-80 µM) or induced GSTs and antioxidant defense (40 µM) are not environmentally relevant; however our goal was to investigate into how daphnids respond to antagonistic mixtures of chemicals (such as HR96 modulators). At this point we propose that atrazine can prove to be a positive control for activation of HR96 in *D. magna*. This will be similar to using high doses of TCPOBOP or dexamethasone that are used as positive controls or reference compounds for CAR/PXR activation (Kawamoto et al., 1999; Pascussi et al., 2000). This will be very relevant for future research in determining some mechanistic pathways associated with HR96 regulation in *D. magna*.
From the background literature available on HR96 regulation in *Drosophila*, we knew that the receptor regulates metabolism and depuration of both toxicants and fats (King-Jones et al., 2006; Sieber and Thummel, 2009, 2012). We were interested in confirming if chemicals that bind to HR96 capable of disrupting energy or lipid allocation in *D. magna*. For our second aim we demonstrated that the HR96 modulators in our research (DHA, LA, atrazine and triclosan) were able to alter starvation stress responses and also induce unique lipidomic signatures in adolescent *D. magna*.

The HR96 inhibitor triclosan represses adult maturation and blocks reproduction during starvation of adolescent daphnids; making the animals enter into a state of senescence. Triclosan is of concern as it has been reported to be one of the most commonly detected pharmaceuticals and personal care product (PPCP) in surface waters and waste water treatment systems (Peng et al., 2013). It has the potential to bioaccumulate via food chain and can affect health of non-target organisms such as freshwater invertebrates (Orvos et al., 2002). Our research shows that triclosan is slowing development of daphnids during starvation, and this is of concern because in the natural ecosystem food conditions are not ideal and organisms may be co-exposed to both chemicals and diet restriction at the same time. Slow or perturbed development induced by triclosan can impact fitness of an aquatic invertebrate population under similar conditions in the environment.

However the other HR96 inhibitor DHA increases starvation survival and does not perturb reproduction. Better reproduction or survival due to DHA pre-exposure points
in the direction that additional food sources may help overcome some of the stress induced by pollutants or food-shortage in the environment. Polar lipid profiles of adolescent daphnids were very different for DHA and LA and similar between triclosan and atrazine; however the ratio of PCs and PEs was perturbed in all groups compared to untreated daphnids. We were able to conclude from this part of our research that toxicants can alter energy utilization and allocation in daphnids by chemical specific means. Further research was needed to understand developmental differences between neonates and adult daphnids and understand why triclosan inhibits adult maturation.

The overarching idea for the next part of our research was to investigate if there are certain exchanges or compromises of specific lipids between adults and neonates and if that could be associated with development or reproduction. We demonstrated the effects of HR96 modulators on starvation survival and reproduction in adult *D. magna* and also survival of neonates born from these adults. The most interesting phenotype was once again associated with the triclosan group, in which adults exposed to triclosan and then starved released neonates that are resistant to starvation possibly because of their ability to enter senescence as once again they did not develop. DHA showed higher reproduction compared to the other HR96 modulators in adult daphnids. We also found out that the percentage of sphingomyelins (SMs) in polar lipids in neonatal daphnids is higher than in adults; with SMs even higher in the DHA and triclosan treated daphnids. Because SMs are precursors to the production of ceramides and sphingosine associated with development and maturation, we concluded that higher SM levels in neonates due to DHA and triclosan pre-exposures was possibly responsible for higher reproduction and
neonatal survival in these two groups respectively. We propose that DHA provides proper dietary lipids for production of ceramides and sphingosines while also reducing SM metabolism so that it could be retained or used for other purposes.

We were able to determine that HR96 is associated with changes in the SM pathway, as we had predicted based on past literature that HR96 may be involved in this pathway. We confirmed that some HR96 regulated genes (magro and sphingomyelinase) are potential biomarkers for this pathway; some of which are downregulated by DHA (HR96 inhibitor) or upregulated by atrazine (HR96 activator). Downregulation of sphingomyelinase by DHA possibly led to higher SM levels in these neonates; but we were not able to demonstrate the same for triclosan. This leaves us with the possibility of future investigations to understand how triclosan exposure may be perturbing other lipid utilization pathways and causing a senescence-like stage in organisms under starvation-stress as our data does not indicate that triclosan works through HR96 in vivo. We need to confirm in future studies which specific lipid metabolism pathway and genes in *D. magna* (of different age groups) are being perturbed by triclosan exposure. Overall our research indicates that specific polar lipids (PC, PE and SM) and HR96-regulated genes (magro and sphingomyelinase) can be used as potential biomarkers to determine toxicant or diet induced stress in a model test organism such as *D. magna*. Future research should aim at determining ceramide and sphingosine levels in neonates born from adults exposed to HR96 modulators to get specific clues as to which of these compounds is perturbed by toxicants or fatty acids.
Lastly we found out that JH analogs did not cause toxicity in larval American lobsters, even though they were tested at concentrations greater than found in the environment. Larval lobsters may not be sensitive to JH analogs like methoprene and pyriproxyfen. We concluded that even though JH analogs are known to inhibit end-stage larval development in crustaceans, they did not have any effect in the larval American lobster irrespective of being tested at significantly higher concentrations. Future research related to this can be attempted to look at the effects of these compounds on other stages of lobsters; considering that larval lobsters may not be a very sensitive stage to determine developmental toxicity of JH analogs. We can also try to confirm in future through analytical techniques the bioavailability of JH analogs to lobsters in salt-water. The fate, transport and bioavailability of pesticides in estuarine environments may be determined by their individual chemical structures, salinity and dissolved organic matter in the water (Weinstein, 2003), leaving us with the possibility to consider these individual parameters in understanding toxicity of JH analogs on larval lobsters.

Overall the results from this work provide further evidence that toxicants and diet can play significant roles in modulating an organism’s sensitivity to other chemicals. These sensitive responses can be manifested as better or poor survival and reproduction, metabolomic alterations and gene expression changes. Some of these effects may be chemical specific or age specific and sometimes both. Organisms respond very differently to chemical-induced stress under limited food resources (such as starvation) versus when provided with additional food sources (such as dietary supplementation like DHA).
A key finding from our study is *D. magna*’s capability in allocating resources towards better survival, under a stressed environment but at the expense of reproductive maturation. This was best demonstrated by the effect of triclosan exposure in our research, where adolescent daphnids pre-exposed to triclosan and then starved did not develop into adulthood and entered a state of senescence and maternal exposure to triclosan led to moderate reproduction (lower than the control group) and interestingly higher survival among neonates born from these triclosan exposed adults. These phenotypes can overall affect the population of the organism if they fail to reproduce and continue to remain in a stressed environment for long.

As an environmental toxicologist my interests specifically lies in understanding key effects associated with the toxicants triclosan and atrazine in this research and summarize separate adverse outcome pathways (Kleinstreuer et al., 2016) (shown in Figure 6.1) associated with these two individual compounds. These proposed pathways may be able to provide directions for future research in understanding how toxicants may interfere with an organism’s xenobiotic and energy metabolism pathways and induce significant effects altering the fitness of the population.
Figure 6.1: Adverse Outcome Pathways of atrazine and triclosan summarizing results and effects observed in this research

References


APPENDICES
APPENDIX A

Supplementary figures from Chapter Two

Appendix A-1: HR96 expression at different ages in *D. magna*. HR96 expression was determined in 2, 4, 7 and 14 day-old daphnids. Data are provided as mean ± SEM. Statistical significance was determined by ANOVA followed by Tukey’s multiple comparison test with Graphpad Prism 4.0. (n = 5 per age group)
APPENDIX B

Supplementary figures from Chapter Three

Appendix B-1: Effects of the fatty acids and toxicants tested on fecundity during 21-day toxicity tests. Reproductive fitness was determined in *D. magna* exposed to (A) DHA, (B) LA, (C) atrazine, and (D) triclosan. Data are presented as mean neonates produced per reproductive female. Statistical significance was measured using one-way ANOVA followed by Fisher’s Least Significant Difference as the post-hoc test with a p-value of 0.05 considered significant. Atrazine significantly reduced reproduction at concentrations at and above 10 μM (C). No other chemicals significantly repressed reproduction.
Appendix B-2: Changes in phosphatidylethanolamine (PE), phosphatidylserine (PS), sphingomyelin (SM) and phosphatidylinositol (PI) species composition among different exposure groups. Changes in percent signal of individual polar lipids following chemical treatment were analyzed by two-way ANOVA (with repeated measures) followed by the Bonferroni post-hoc test. Statistical differences in each of the polar lipid species are noted in the table below. ‘a’ indicates UT vs DHA, ‘b’ is UT vs LA, ‘c’ is UT vs Atr, ‘d’ is UT vs Tric, ‘e’ is DHA vs Atr, ‘f’ is LA vs Atr and ‘g’ is LA vs Tric (only letter = p<0.01, * indicates p<0.001)
Appendix B-3: Relative concentrations of phosphatidylethanolamine (PE)-based polar lipids following exposure to different chemicals for 4 days. Changes in the percent signal of PE based polar lipids were determined by one-way ANOVA followed by Tukey’s post-hoc test (n = 5). Each dot represents an individual daphnid, and the mean is represented by a horizontal line. An ‘a’ indicates statistical differences from all other treatments, ‘b’ is different from untreated group, ‘c’ is different from DHA, ‘d’ is different from LA, ‘e’ is different from atrazine and ‘f’ is different from triclosan (p<0.05, * p<0.01 and **p<0.001).
Appendix B-4: Changes in phosphatidylcholine (PC) species composition among daphnids exposed to different chemicals. Changes in percent signal of individual PC species following chemical treatment were analyzed by two-way ANOVA (with repeated measures) followed by the Bonferroni post-hoc test. Statistical differences are described below the graph. Statistical differences between different groups are noted with letters; ‘a’ indicates UT vs DHA, ‘b’ is UT vs LA, ‘c’ is UT vs Atr, ‘d’ is UT vs Tric , ‘e’ is DHA vs Tric, ‘f’ is LA vs Atr and ‘g’ is LA vs Tric (only letter = p<0.01, * indicates p<0.001).
### Differences between groups

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Appendix B-5: Relative concentrations of phosphatidylcholine (PC)-based polar lipids following exposure to different chemicals for 4 days. Changes in the percent signal of PC based polar lipids were determined by one-way ANOVA followed by Tukey’s post-hoc test (n = 5). Each dot represents an individual daphnid, and the mean is represented by a horizontal line. An ‘a’ indicates statistical differences from all other treatments, ‘b’ is different from untreated group, ‘c’ is different from DHA, ‘d’ is different from LA, ‘e’ is different from atrazine and ‘f’ is different from triclosan (p<0.05, * p<0.01 and **p<0.001).
Appendix B-6: Hierarchical Clustering (HCL) was used to cluster and visualize significant changes in the concentrations of individual lipids using MultiExperiment Viewer (MeV). DHA forms its own clade and atrazine, triclosan, and untreated daphnids cluster together.
Appendix B-7: Relative concentrations of phosphatidinositol (PI), phosphatidylserine (PS), and sphingomyeline (SM) -based polar lipids following exposure to different chemicals for 4 days. Changes in the percent signal of individual species were determined by one-way ANOVA followed by Tukey’s post-hoc test (n = 5). Each dot represents an individual daphnid, and the mean is represented by a horizontal line. An ‘a’ indicates statistical differences from all other treatments, ‘b’ is different from untreated group, ‘c’ is different from DHA, ‘d’ is different from LA, ‘e’ is different from atrazine and ‘f’ is different from triclosan (p<0.05, * p<0.01 and **p<0.001).
APPENDIX C

Supplementary figures from Chapter Four

Table Appendix C-1: Quantity of each lipid type found in the polar lipids extracted from treated and untreated neonatal *D. magna* (per animal). Data presented as mean nmol lipid/daphnid +/- standard error. Statistical differences determined by two-way ANOVA followed by Fisher’s LSD post-hoc test (p<0.05, *p<0.01, **p<0.001) (n=5).

‘a’ refers to treatments different than UT
‘b’ refers to treatments different than DHA
‘c’ refers to treatments different than LA
‘d’ refers to treatments different than Atr
‘e’ refers to treatments different than Tric

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Appendix C-2: (A) Hierarchical Clustering (HCL) was used to cluster and visualize significant changes in the percentage signal of individual lipids in the adult daphnids using MultiExperiment Viewer (MeV). One-way ANOVA (p<0.01) was used to identify significantly altered lipid species. (B): Principle component analysis (PCA) demonstrates associations between chemical exposures and lipid profiles in adult *D. magna*. Variability among the polar lipids was observed following chemical treatment.
Appendix C-3: (A) Percent survival of daphnids exposed to ceramidase inhibitor Carmofur for 21-days: Percent survival was not altered in this study. (B) Percent survival of daphnids exposed to sphingomyelinase inhibitor GW4869 for 21-days: Percent survival was not altered in this study. Statistical analysis was performed using Fisher’s 2x2 tail test.