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Chronic Exposure to Low Molecular Weight Polycyclic Aromatic Hydrocarbons Promotes  
Metabolic Inflammation in Vertebrate and Invertebrate Systems

by

Asia S. Bright

Under the Direction of Bingzhong Xue, PhD

A Dissertation Submitted in Partial Fulfillment of the Requirements for the Degree of

Doctor of Philosophy

in the College of Arts and Sciences

Georgia State University

2023

## ABSTRACT

2-naphthol is a low molecular weight polycyclic aromatic hydrocarbon and ubiquitous air pollutant. Recent epidemiological reports suggest that exposure to individual low molecular weight polycyclic aromatic hydrocarbons is correlated with increased incidences of childhood obesity. Yet, most studies focus on the health effects of either high molecular weight molecules or polycyclic aromatic hydrocarbon mixtures. This research explores the potential obesogenic impact of 2-naphthol in cell and animal models. To assess the *in vivo* effects of 2-naphthol, we monitored life fitness traits and lipid accumulation in the invertebrate *Daphnia magna* across development. Results showed that *D. magna* directly exposed to 2-naphthol demonstrated delayed reproductive development, produced fewer offspring, and accumulated more lipids than *D. magna* that were not exposed to 2-naphthol. Analysis of lipid accumulation by Nile Red staining revealed a dose-dependent increase in overall lipid quantity. To confirm these findings, we also examined the effect of 2-naphthol on lipid metabolism and inflammation *in vitro*. We employed 3T3-L1 and BAT1 mammalian cell lines to model white and brown adipocytes, respectively, alongside a murine macrophage cell line (RAW264.7). Cells were challenged with either acute or chronic 2-naphthol treatments, then differentiated adipocytes were assessed for changes in adipogenesis, lipid accumulation, and isoproterenol-stimulated thermogenesis. Additionally, LPS-induced inflammation was evaluated in both adipocytes and macrophages. 2-naphthol exposure increased the expression of key adipogenic and lipogenic genes, while lipolytic gene expression decreased in both chronically treated adipocyte lines. Further, 2-naphthol exposure suppressed isoproterenol-stimulated thermogenic gene expression in BAT1 brown adipocytes. In consistence, adipocytes exposed to 2-naphthol and stained with BODIPY and Oil Red O showed an increase in lipid accumulation. Additionally, adipocytes and macrophages chronically exposed to 2-naphthol

showed an up-regulation in mRNA expression of major inflammatory cytokines, such as *Tnfa*, *Il-1 $\beta$* , and *Il-6*. Overall, these findings support previous research that indicates 2-naphthol is a possible obesogen that may contribute to the development of obesity and associated metabolic disorders via modulating lipid metabolism and thermogenesis in adipocytes, and inflammation in both adipocytes and macrophages.

INDEX WORDS: Polycyclic aromatic hydrocarbon, *Daphnia*, Adipocyte, Lipid metabolism, Inflammation, Differentiation, Adipogenesis

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2023

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Metabolic Inflammation in Vertebrate and Invertebrate Systems

by

Asia Bright

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May 2023

## DEDICATION

To my mother, father, and grandparents, who have always been my greatest supporters and role models. Your love and guidance have been invaluable to me, and I am forever grateful.

To my superman, Kal-El, and my rambunctious new baby boy, Zariah. You bring joy and light to my life every day. Your endless energy and curiosity inspire me to be a better person (or at least try to keep up with you). Thank you for being the light of my life and always reminding me of my capabilities. (See Appendix A)

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**LIST OF ABBREVIATIONS**

2-NAP	2-Naphthol
AMP	Antimicrobial Peptide
ANOVA,	Analysis Of Variance
aP2/FABP4	Adipocyte Protein 2/Fatty Acid Binding Protein 4
ATM	Adipose Tissue Macrophage
ATP	Adenosine Triphosphate
BAT	Brown Adipose Tissue
BDM	Bat1 Differentiation Media
BEM	Bat1 Expansion Medium
BMI,	Body Mass Index
BMM	Bat1 Maintenance Medium
BODIPY	4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3A,4A-Diaza-S-Indacene
BPA,	Bisphenol A
C/EBP	Ccaat/Enhancer-Binding Protein
CCL1	C-C Chemokine Ligand 1
CCL2	C-C Chemokine Ligand 2
CD11	Cluster Of Differentiation 11
CD206,	Cluster Of Differentiation 206
CD301,	Cluster Of Differentiation 301
CREB	Cyclic Amp-Response Element-Binding Protein
CS	Calf Serum
CXCL1,	C-X-C Chemokine Ligand 1
CXCL12,	C-X-C Chemokine Ligand 12
DEHP,	Di(2-Ethylhexyl) Phthalate
DEX	Dexamethasone
DM	Differentiation Media
DMEM	Dulbecco's Modified Eagle's Medium
ELISA	Enzyme-Linked Immunosorbent Assay
EM	Expansion Medium
EtOH	Ethanol
F0	Filial 0 (Parental Generation)
F1	First Filial (1st generation of offspring)
FBS	Fetal Bovine Serum
HMW	High Molecular Weight
IBMX,	3-Isobutyl-1-Methylxanthine
IFN	Interferon

IL-6	Interleukin 6
IL- $\beta$	Interleukin Beta
IRS2,	Insulin Receptor Substrate 2
ISO	Isoproterenol
LMW	Low Molecular Weight
LPS	Lipopolysaccharide
MCP	Monocyte Chemoattractant Protein
NOS2	Nitric Oxide Synthase 2
ORO,	Oil Red O
PAH,	Polycyclic Aromatic Hydrocarbon
PCR	Polymerase Chain Reaction
PGC1 $\alpha$	Peroxisome Proliferator-Activated Receptor Gamma Coactivator 1 Alpha
PKA	Protein Kinase A
RXR	Retinoid X Receptor
SEM	Scanning Electron Microscopy
SREBP1	Sterol Regulatory Element-Binding Protein 1
T <sub>3</sub>	Triiodothyronine
TNF $\alpha$	Tumor Necrosis Factor Alpha
UCP1	Uncoupling Protein 1
WAT	White Adipose Tissue
$\beta$ AR	Beta Adrenergic Receptor



## 1 INTRODUCTION AND LITERATURE REVIEW

### 1.1 Obesity and adipose tissue biology

Obesity is a chronic condition where abnormalities in adipose tissue function lead to excessive tissue growth and chronic inflammation. Genetics, lifestyle, and environmental pressures can influence this multifaceted disorder. Physical inactivity, diet, medication, and exposure to adipogenic pollutants are just a few factors that contribute to the obese phenotype in humans. The prevalence of obesity throughout the developing world has increased significantly over the past few decades (Flegal et al., 2016). Individuals diagnosed with obesity are at a higher risk of developing metabolic syndrome and related metabolic disorders, including diabetes, dyslipidemia, atherosclerosis, hypertension, or even cancer (Fantuzzi, 2005). At the cellular level, obesity is characterized by excessive lipid droplet accumulation within the adipocytes, commonly known as fat cells, of white adipose tissue (hypertrophy) alongside increased fat cell number (hyperplasia) (Schäffler & Scholmerich, 2010). Establishing adipocyte population size and development is critical during childhood (Boulton et al., 1978). Once we reach adulthood, we usually maintain a constant number of adipocytes. Comparing obese adults to their non-obese counterparts shows increased incidences of hyperplasia and hypertrophy (Knittle et al., 1979; Salans et al., 1973). When trying to combat obesity, adults can reduce the size of existing adipocytes, but it is almost impossible to reduce the total number of fat cells.

The adipose organ is a complex structure composed primarily of two types of tissues, white (WAT) and brown adipose tissue (BAT). These tissues function as fat depots, endocrine regulators, and inflammatory mediators. Distinct structural and biochemical properties between the two primary adipose tissues arise from their cellular origin, gene activity, and metabolic profiles. WAT is the

most abundant of the two tissues and the body's primary energy storage site. In contrast, the primary role of BAT is energy expenditure via nonshivering thermogenesis (Berry et al., 2013). It was initially believed that BAT predominantly functions in the early stages of development (Rosell et al., 2014; Ruiz-Ojeda et al., 2016a), whereas WAT is far more pervasive and biologically active in adult humans. However, recent studies have demonstrated that BAT is present and functional in adults (van den Berg et al., 2017). Adipocytes are the primary cell type found within adipose tissues (Ali et al., 2013). They originate from multipotent mesenchymal progenitor cells present in the adipose tissue stroma. Their primary function is storing energy in the form of lipid droplets, lipid-rich cellular organelles that regulate the storage and hydrolysis of neutral lipids. Adipocytes use stored lipids for energy, steroid synthesis, or membrane formation. Lipid droplet accumulation is a normal function in fat cells. However, excessive lipid droplet accumulation within adipocytes often indicates metabolic dysfunction.

Disturbances in adipocyte function often happen during adipogenesis, the process by which committed preadipocytes differentiate into mature adipocytes. Adipogenesis is marked by several phases: exponential growth in cellular population, cell cycle arrest, clonal expansion, and terminal differentiation. This process is characterized by significant alterations in cellular genotype and phenotype. Undifferentiated preadipocytes have a fibroblast-like structure. After differentiation, they transform into round lipid-filled adipocytes. The transcriptional pathways that direct adipogenesis are highly regulated and have been extensively studied. This process is carried out through a series of signaling cascades mediated by interactions between key transcriptional regulators. These events stimulate the expression of a battery of adipogenic genes necessary to induce terminal differentiation and give rise to the mature adipocyte phenotype. The advancement

of traditional cell models and biochemical methodologies has enhanced collective knowledge surrounding metabolic dysfunctions and associated pathologies. Murine embryonic cell lines, such as 3T3-L1 and immortalized BAT1 brown adipocyte cell lines that can be chemically induced to differentiate into white or brown adipocytes, respectively, are pertinent to our understanding of adipose tissue biology (Ruiz-Ojeda et al., 2016b). 3T3-L1 cells are among the more popular *in vitro* models used to analyze white adipocyte differentiation. This is primarily because the cellular and molecular events that occur during the transition from undifferentiated fibroblast-like preadipocytes into mature adipocytes have been extensively characterized. BAT1 cells, on the other hand, are a suitable brown adipocyte model to study various mechanisms that regulate thermogenesis and their potential contributions to the regulation of energy expenditure and the development of obesity.

To initiate adipogenesis *in vitro*, preadipocytes are treated with a hormonal cocktail following the exponential growth phase (Darimont & Macé, 2003; Gregoire, 2001; Ruiz-Ojeda et al., 2016b). For white adipocytes, this cocktail includes insulin, isobutylmethylxanthine, and dexamethasone. An equivalent formula is used for brown adipocyte differentiation, with the addition of indomethacin and triiodothyronine ( $T_3$ ). Upon induction of differentiation, preadipocytes are prompted to enter the cell cycle, go through one or two mitotic divisions, and permanently withdraw from the cell cycle. In the early phases of differentiation, proliferating cells produce the  $\beta$  and  $\delta$  isoforms of the CCAAT/enhancer binding protein (C/EBP) family of transcription factors. The glucocorticoid dexamethasone facilitates C/EBP $\delta$  transcription, and isobutylmethylxanthine, a phosphodiesterase inhibitor, induces C/EBP $\beta$  transcription (Nakagami, 2013; Ruiz-Ojeda et al., 2016b). Meanwhile, insulin acts as a growth factor, enhancing adipogenesis. C/EBP $\beta$  and C/EBP $\delta$

trigger the production of peroxisome proliferator-activated receptor- $\gamma$  (PPAR $\gamma$ ) and C/EBP $\alpha$ , key transcription factors involved in the growth arrest phase required for adipocyte differentiation (Ahmadian et al., 2013). PPAR $\gamma$  and C/EBP $\alpha$  can act as coactivators or bind other endogenous nuclear receptors, such as the 9-cis retinoic acid receptor (RXR), to transactivate fat-specific genes responsible for the mature adipocyte phenotype. PPAR $\gamma$  is a member of the nuclear receptor superfamily and is both necessary and sufficient for adipogenesis (Kajimura et al., 2010; Metzger et al., 2005). PPAR $\gamma$  is not only crucial for adipogenesis but is also required for the maintenance of the differentiated state. C/EBP $\alpha$  induces many adipocyte genes directly, and *in vivo* studies indicate an essential role of this factor in the development of adipose tissue. During the differentiation process, it is expected to see changes in the expression of hallmark genes such as *Ppar $\gamma$*  and *Cebpa*, markers of adipocyte differentiation; the lipogenesis marker fatty acid synthase (*Fasn*); lipolysis markers hormone-sensitive lipase (*Hsl*) and adipose triglyceride lipase (*Atgl*); and the adipokine adiponectin (*Adipoq*) (Gregoire, 2001; Klaus et al., 1995; Ruiz-Ojeda et al., 2016b).

BAT is distinguished from WAT by various morphological and functional characteristics. For example, the lipid droplets of brown adipocytes are multilocular as opposed to the unilocular droplet found in white adipocytes. Functionally, BAT specializes in energy dissipation via a process called non-shivering thermogenesis. Furthermore, during differentiation, brown adipocytes show significant increases in the expression of lipolytic and thermogenic markers, and similar expression increases in adipogenic and lipogenic markers when compared to white adipocytes. In response to cold exposure or sometimes dietary changes, mitochondria of brown adipocytes will produce the thermogenic marker, uncoupling protein-1 (UCP1) (Seale et al., 2009). The nuclear receptor, PPAR $\gamma$  coactivator 1 alpha (*Pgc1 $\alpha$* ), another marker of thermogenesis,

regulates *UCPI* (Uldry et al., 2006). The expression of *Pgc1 $\alpha$*  promotes brown adipocyte differentiation and encourages mitochondrial biogenesis (Lin et al., 2005). BAT is regulated and innervated by neurons of the sympathetic nervous system (SNS). The SNS is stimulated by activating various adrenergic  $\beta$ -adrenergic receptors ( $\beta$ ARs) (Cannon & Nedergaard, 2004). Functionally, the  $\beta$ ARs are widely known to regulate many different biological events, such as cellular proliferation, differentiation, and diet-induced thermogenesis. Activation of adrenergic receptors will stimulate lipolysis and the subsequent release of fatty acids from adipose tissue depots (Garretson et al., 2016). When a catecholamine such as norepinephrine binds to its receptor  $\beta$ AR, the downstream signaling activates the enzyme adenylyl cyclase, which converts adenosine triphosphate (ATP) into the second messenger, cyclic AMP (cAMP). In turn, cAMP will bind the inhibitory regulatory subunit of protein kinase A (PKA) (Grisouard et al., 2010; Kajimura et al., 2010). cAMP binding to PKA will dissociate the inhibitory subunit from the PKA complex, activating PKA and allowing it to phosphorylate downstream targets. PKA can bind transcriptional factors to facilitate gene transcription or other kinases to continue signaling, including cAMP response element-binding protein (CREB) and hepatic phosphorylase kinase. *PGC1 $\alpha$*  is activated downstream of the PKA/CREB signaling cascade (Dowal et al., 2017).

*In vitro* preadipocyte models, like the 3T3-L1 and BAT1 cell lines, allow more in-depth analyses of the biological mechanisms that direct adipogenesis and thermogenic gene expression. Changes in gene expression at the mRNA level can be monitored using standardized techniques like real-time quantitative reverse transcription polymerase chain reaction (qRT-PCR). Researchers can use established protocols to observe changes in adipocyte function in response to exogenous or endogenous changes.

## **1.2 Adipose tissue inflammation**

Emerging research has revealed the dynamic nature of adipose tissues as a major endocrine organ and its involvement in immune function (Ahima & Flier, 2000; Chan et al., 2019; Omran & Christian, 2020; Zhang et al., 2015). Adipose tissues are populated primarily by adipocytes, among other cell types endowed with remarkable plastic properties, including immune cells, fibroblast, endothelial cells, and stem cells (Kahn et al., 2019). Physiological changes within adipose tissue microenvironments, such as nutrient availability, environmental factors, or even shifts in ambient temperature, can influence an organism's overall metabolic health. Recent investigations into the complex nature of adipose tissues show that both WAT and BAT have active roles in maintaining energy homeostasis and modulating immunoregulatory mechanisms (Chan et al., 2019; Fantuzzi, 2005; Schäffler & Schölmerich, 2010).

The plasticity of adipose tissue makes it responsive to various endogenous and exogenous factors. The cells that make up adipose tissue produce innumerable compounds involved in inflammation, adipogenesis, metabolism, and overall metabolic homeostasis (Fantuzzi, 2005). Cross-talk between adipocytes and macrophages is necessary for adipose tissue maintenance. Like macrophages and other immune cells, adipocytes can express a variety of chemokines, cytokines, and hormones (Kim & Bajaj, 2014). To begin assessing the role of environmental influences, like 2-NAP, within the confines of adipose tissue inflammation, we must understand the relationship between adipocytes and macrophages. This section will focus on the role of inflammation in the emergence of obesity-associated inflammation, including the involvement of innate immune

receptors (TLRs) and inflammatory mediators such as tumor necrosis factor  $\alpha$  (*Tnfa*), Interleukin- $1\beta$  (*Il-1\beta*), and *Il-6*.

### ***1.2.1 Adipocytes***

Many metabolic disorders are linked to perturbations within the WAT. White adipocytes are no longer viewed as dormant cells exclusively used for energy storage. Instead, they are now considered critical modulators of immunological and inflammatory processes. Similar to innate immune cells, white adipocytes can produce inflammatory proteins like TNF $\alpha$ , Il- $1\beta$ , and Il-6 (Hotamisligil et al., 1995; Poulain-Godefroy & Froguel, 2007; Schäffler & Scholmerich, 2010). The effects of proinflammatory cytokines on WAT function are often multi-dimensional. A single molecule may exhibit biphasic characteristics, which may be advantageous under certain conditions and toxic in others. A myriad of components within a tissue's surrounding microenvironment can affect how a single molecule influences the health of an organ system, including micronutrient concentration, the presence or absence of binding partners, and exogenous factors.

The literature is replete with contradictory information about the role of inflammatory mediators in adipose tissue homeostasis. Elevated proinflammatory cytokine levels characterize the pathogenesis of most metabolic diseases, and throughout the literature, adipocytes are described as the drivers of metabolic inflammation. Adipocytes from obese subjects after weight loss show a reduction in monocyte the monocyte chemoattractant protein-1 (MCP-1) production (Christiansen et al., 2005). White adipocytes facilitate cellular recruitment by producing chemokines such as CXCL1, CXCL12, and CCL2 (Chirumbolo et al., 2014; Poulain-Godefroy &

Froguel, 2007). They may also influence transient macrophage polarization through the production of inflammatory cytokines. Additionally, adipocytes react to many of the same inflammatory, recruitment, and growth mediators in a paracrine or autocrine fashion (Ouchi et al., 2011). These findings imply that white adipocytes can modify macrophage inflammatory state as demonstrated by pronounced alterations in their gene expression profiles. 3T3-L1 cells challenged with LPS show increased cytokine and chemokine production, including IL-6. Brown fat cells are distinguished from white fat through mitochondrial markers (Omran & Christian, 2020). A survey of the literature illustrated that BAT extracted from obese subjects or under impaired adrenergic signaling shows a whitening phenotype and is characterized by increased inflammation (Cinti et al., 1997; Jespersen et al., 2020; Kotzbeck et al., 2018; Shimizu et al., 2014). These results indicate that BAT is important in maintaining metabolic homeostasis. In obese mice, areas typically populated by brown adipocytes contain unilocular cells, unlike the classical multilocular adipocytes present in BAT (Cinti et al., 1997). Interestingly, obesity-driven increases in TNF $\alpha$  expression in mice heightened the apoptosis rate of brown fat cells (Nisoli et al., 2000).

Understanding the complexity of the mechanisms and regulators involved in adipose tissue development and homeostasis is vitally important if we want to explore the influence of exogenous factors. Many examples throughout the literature show that the inflammatory potential of endogenous and exogenous molecules is conditional to the surrounding environment. Contributing factors to consider when evaluating obesogenic potential include exposure length (acute vs. chronic), concentration, developmental stages, and the subject's health status. For example, preadipocytes demonstrate increased susceptibility to inflammatory stimuli, producing higher cytokine levels in response to LPS stimulation compared to mature adipocytes (Poulain-Godefroy



& Froguel, 2007). This effect is also evident *in vivo*, as WAT from LPS-challenged mice was shown to be the primary site of IL-6 production (Starr et al., 2009). We also see examples of duplicity where cytokines upregulated during obesity can exhibit both lipolytic and adipogenic effects simultaneously. Available evidence shows that NOS2 can regulate lipolysis and contribute to preadipocyte maturation (Engeli et al., 2004). NOS2 and other cytokines can also modulate energy storage and utilization mechanisms by binding co-receptors expressed on the surface of white adipocytes (Bickel et al., 2009; Kahn et al., 2019; Munro et al., 2020). These examples provide a glimpse into the intricacy of the molecules released by adipocytes and other cells within WAT. Interestingly, some findings showcase the necessity of proinflammatory mediators to maintain normal adipose tissue development and function. (Wernstedt Asterholm et al., 2014) discovered that acute inflammation is essential to successful adipogenesis. Interestingly, acute instances of inflammation did not adversely affect fat mass or glucose tolerance (Wernstedt Asterholm et al., 2014). There is even evidence of differentiating adipocytes functioning as modulators of innate immunity by producing antimicrobial peptides (Zhang et al., 2015). Therefore, it is critical to consider adipose tissue plasticity when designing the conceptual framework for screening and evaluating potential obesogens.

### ***1.2.2 Macrophages***

Exogenous compounds that alter macrophage activation profiles can influence macrophage polarization and disrupt signaling pathways that mediate metabolic homeostasis. We can exploit these mechanisms to understand how toxic compounds influence immunoregulatory systems that also coregulate metabolic activity. The macrophage phenotype is defined by the combinatorial expression of proinflammatory (M1) or anti-inflammatory (M2) surface receptors and signature

cytokine profiles (Remmerie & Scott, 2018). M1 macrophages express CD11b, CD11c, F4/80, and secrete inflammatory cytokines. M2 macrophages also express the classical macrophage markers CD11b and F4/80. M2 macrophages also express CD301, CD206, and secrete anti-inflammatory cytokines. It should be noted that macrophage polarization is a transient event. Most macrophages exist somewhere between extreme M1 and M2 phenotypes. Nonetheless, the chemical profiles associated with M1-like or M2-like phenotypes are divergent enough to influence distinct signaling pathways and protein production. These phenotypes can be linked to metabolic homeostasis.

The WAT of obesity-resistant animal models is primarily populated by M2-like macrophages, in contrast to the WAT of obese subjects, typically comprised of M1-like macrophages (Lumeng et al., 2007, 2008). Continuous macrophage infiltration within adipose tissues induces chronic low-grade inflammation associated with metabolic dysfunctions, which is then propagated by the hyper-stimulation of resident adipose tissue macrophages (ATMs) by endogenous and/or exogenous stimuli. Neighboring adipocytes will produce chemokines that further promote monocyte recruitment into adipose tissues and continual activation of ATMs—resulting in a seemingly endless feedback loop cycling between lipid accumulation and chronic low-grade inflammation. Additionally, macrophage population size within the adipose tissues is directly related to adiposity (Weisberg et al., 2003). Immunologists generally recognize that the M2 phenotype promotes an environment that increases insulin sensitivity and prevents insulin resistance (McNelis & Olefsky, 2014). Macrophages are highly plastic cells, allowing them to transform their phenotype in response to environmental shifts. Increasingly, studies are revealing the role of lipid metabolism in the immunomodulation of macrophages, jointly with its influence

on the progression of related metabolic disorders (including obesity, type II diabetes, and liver diseases) (Cao et al., 2014; McNelis & Olefsky, 2014). Glucose is the preferred metabolic substrate for many cellular processes, especially for high metabolic activities such as innate immune activation and function. Pathways targeted by macrophage products include those related to lipid homeostasis, glucose utilization and storage, and other metabolic processes (Remmerie & Scott, 2018). An unexpected shift (maybe due to environmental exposures) from an M2 to an M1 phenotype during a diseased state is a sufficient enough stimulus to promote insulin resistance and impair local glucose uptake and utilization (Fantuzzi, 2005).

Macrophages are critical regulators of innate immunity and are essential to balancing pro-inflammatory and anti-inflammatory conditions of tissues throughout the body. Exploring the effects of obesogens on macrophage function is crucial to uncovering cellular mechanisms that trigger the onset of proinflammatory conditions during obesity development.

### **1.3 Low molecular weight polycyclic aromatic hydrocarbons and obesity**

With the prevalence of obesity growing steadily, the research focus has shifted to identifying the role environmental obesogens play in the pathophysiology and development of the disease. The adipogenic effects of most obesogens likely begin during early fetal and infant stages (Janesick & Blumberg, 2011). Long-term exposure to endocrine-disrupting chemicals during formative developmental periods often disrupts genetic and metabolic processes responsible for the normal development of adipose tissues. The potential obesogenic effects of polycyclic aromatic hydrocarbons (PAHs) are gaining recognition for their connections to the increasing prevalence of human metabolic dysfunctions (Liu et al., 2013). Previous animal studies have explored the

relationships between PAH exposure and increased adiposity and identified potential mechanisms involved. The offspring of mice exposed to PAHs via inhalation during pregnancy exhibited increases in fat mass, body weight, and expression of adipogenic genes (Z. Yan et al., 2014). Another group observed a similar pattern of results, which showed that mice subjected to prenatal exposure of diesel exhaust produced offspring with higher insulin resistance, increased adipose tissue inflammation, and upregulated inflammatory cytokine expression (Bolton et al., 2014). Fetal exposure to the HMW-PAH, benzo(a)pyrene, significantly increases adipose tissue size in developing mice. Benzo(a)pyrene also promotes adipogenesis and lipid accumulation by epigenetically activating PPAR $\gamma$  (Z. Yan et al., 2014). However, available data is limited, and no previous studies have focused on human exposure to individual PAH compounds (European Commission, 2002).

### ***1.3.1 Physicochemical properties of naphthalene and its metabolite 2-naphthol***

As mentioned earlier, PAHs are a class of heterocyclic xenobiotics compounds produced as byproducts of burning organic matter. They are categorized based on the number of fused aromatic rings in the parent structures. There are two general classifications of PAHs: 1) low-molecular-weight (LMW) PAHs have 2 to 3 aromatic rings (e.g., naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, and anthracene), and 2) high-molecular-weight (HMW) PAHs with 4 or more aromatic rings (e.g., pyrene, benzo(a)pyrene, and dibenz[a,h]anthracene). PAH toxicity and environmental deposition are influenced by physiochemical factors such as structure, molecular weight, and solubility (Nisbet & LaGoy, 1992; Zhu et al., 2009). HMW-PAHs usually exist bound to particulate matter, whereas LMW-PAHs remain in the atmosphere as vapors (Baek et al., 1991; Cautreels & van Cauwenberghe, 1978). The physical and chemical attributes of PAH parent compounds identified as target pollutants by the US EPA are summarized in Table 1.

**Table 1.1 Physical and Chemical Attributes of PAH Parent Compounds Classified as Priority Pollutants.**

Nomenclature	Structure	Chemical Formula	Exact Mass (g)	Molecular weight (g/mol)	Melting Point [K]	Boiling Point [K]	Solubility in Water (mg/L) <sup>a,b</sup>	Phase Distribution <sup>c</sup>	EPA <sup>d</sup>
Naphthalene		C <sub>10</sub> H <sub>8</sub>	128.06	128.17	261.08	504.82	31.2000	Gas	C
Acenaphthylene		C <sub>12</sub> H <sub>8</sub>	152.06	152.20	335.12	567.59	3.9300	Gas	D
Acenaphthene		C <sub>12</sub> H <sub>10</sub>	154.08	154.21	334.36	545.89	3.9000	Gas	D
Fluorene		C <sub>13</sub> H <sub>10</sub>	166.08	166.22	342.87	546.71	1.8900	Gas	D
Anthracene		C <sub>14</sub> H <sub>10</sub>	178.08	178.23	351.38	600.48	0.0434	Particle gas	D
Phenanthrene		C <sub>14</sub> H <sub>10</sub>	178.08	178.23	351.38	600.48	1.1500	Particle gas	D
Fluoranthene		C <sub>16</sub> H <sub>10</sub>	202.08	202.26	425.42	645.02	0.2600	Particle gas	D
Pyrene		C <sub>16</sub> H <sub>10</sub>	202.08	202.26	425.42	645.02	0.0770	Particle gas	D
Benz[a]anthracene		C <sub>18</sub> H <sub>12</sub>	228.09	228.29	441.68	672.36	0.0100	Particle	B2
Chrysene		C <sub>18</sub> H <sub>12</sub>	228.09	228.29	441.68	672.36	0.0026	Particle	B2
Benzo[a]pyrene		C <sub>20</sub> H <sub>12</sub>	252.09	252.32	515.72	715.92	0.0023	Particle	B2
Benzo[b]fluoranthene		C <sub>20</sub> H <sub>12</sub>	252.09	252.32	515.72	715.92	0.0012	Particle	B2
Benzo[k]fluoranthene		C <sub>20</sub> H <sub>12</sub>	252.09	252.32	515.72	715.92	0.0008	Particle	B2
Benzo[g,h,i]perylene		C <sub>22</sub> H <sub>12</sub>	276.09	276.34	589.76	759.48	0.0003	Particle	D
Indeno[1,2,3-cd]pyrene		C <sub>22</sub> H <sub>12</sub>	276.09	276.34	589.76	759.48	0.0620	Particle	B2
Dibenz[a,h]anthracene		C <sub>22</sub> H <sub>14</sub>	278.11	278.35	531.98	743.26	0.0005	Particle	B2

Chemical structures for LMW-PAHs are colored red. Structures for HMW-PAHs are colored black.

<sup>a</sup> (Agency for Toxic Substances and Disease Registry (ATSDR), 1995, 2005)

<sup>b</sup> Chemical structures and chemical property values were generated using ChemDraw v.220.0.22 software (ChemDraw, 2022).

<sup>c</sup> (Patel et al., 2020)

<sup>d</sup> (J. Yan et al., 2004); EPA carcinogenic classification: A, human carcinogen; B1 and B2: probable human carcinogen; C, possible human carcinogen; D, not classifiable as to human carcinogenicity; E, evidence of non-carcinogenicity for humans. (EPA, 1982, 1984)

The intrinsic properties of LMW-PAHs make them more environmentally prevalent than their HMW counterparts. As a result, the lipophilic LMW-PAH are more likely to accumulate in fatty tissues (e.g., adipose, liver, and kidneys) at higher rates than other PAHs. PAH parent compounds are generally non-reactive, and most adverse health effects can be attributed to their metabolically modified derivatives (Pelkonen & Nebert, 1982). Naphthalene is structurally the simplest PAH (made up of only two benzene rings). Parent PAH compounds can be modified in many ways based on where they are metabolized in the body and what metabolic reactions are taking place. The double bonds within the benzene rings of parent hydrocarbons are predominantly converted to hydroxylated derivatives. Intermediate products formed during the metabolism of parent PAH compounds are responsible for malignant transformations within mouse prostate cells (Grover et al., 1971). Hydroxylated PAH (OH-PAH) derivatives have OH groups added to one or more of the carbons of the aromatic rings. OH-PAH isotypes are structurally homologous to estrogen and have been shown to bind different estrogen receptors (Schultz & Sinks, 2002; Sievers et al., 2013). Naphthalene's biologically active metabolite, 2-naphthol (2-NAP), is the focus of this study and a great starting point for evaluating the effects of LMW-PAHs.

### ***1.3.2 Sources, routes, and epidemiological evidence of human exposure***

Human exposure to polycyclic aromatic hydrocarbons (PAH) has significantly increased over the past few decades. Most PAHs enter the environment as atmospheric pollution from the incomplete combustion of coal, fossil fuels, oil, and gas. Subsequently, the primary route of human exposure is inhalation (Agency for Toxic Substances and Disease Registry (ATSDR), 1995, 2005). Exposure to anthropogenic LMW-PAH sources (automobile exhaust, cigarette smoke, and

industrial emissions) is rising in tandem with urban and industrial advancements; however, many studies focus on the obesogenic effects of PAH mixtures or HMW-PAHs. Exposure to PAHs can also occur via ingesting contaminated food and drinking water. Numerous dietary items have been shown to contain PAHs, including unprocessed and processed grains, root vegetables, oils, and cooked (European Commission, 2002). Compounds such as naphthalene can be formed when cooking meat at high temperatures (Lewtas, 2007). Dermal exposure is another possible pathway for human contact. Exposure through skin adsorption is usually specific to industrial workers or individuals who come in direct contact with high concentrations of PAHs, ash, coal, or tar.

Data from recent epidemiological studies suggest significant correlations between PAH exposure and incidences of obesity and metabolic inflammation. Measurements of urinary biomarkers in a representative sample of US adults revealed associations between LMW-PAH exposure and increased concentrations of biological markers associated with chronic inflammation (Alshaarawy et al., 2013; Ferguson et al., 2011). Likewise, we see that occurrences of childhood obesity are positively linked to increasing concentrations of individual LMW-PAH urinary metabolites, such as 2-NAP (Scinicariello & Buser, 2014). Consistent with animal studies examining prenatal PAH exposures, Rundle et al 2012Rundle et al 2012Rundle et al 2012Rundle et al 2012, found correlations between human prenatal PAH exposure and increased adiposity in mothers and children living in New York City – an urbanized metropolitan city with high levels of air pollution. A recurrent pattern in many of these epidemiological observations is the propensity of stronger correlations between LMW-PAH exposure and obesity-related pathologies compared to HMW-PAH exposures (Alshaarawy et al., 2013; Scinicariello & Buser, 2014). The same study showed specific correlations between naphthalene metabolites and rises in BMI and waist circumference

measurements. 2-NAP was also found to be the most concentrated LMW-PAH in urine samples from children categorized as obese. Further supporting evidence describes positive relationships between air pollution and the development of related diseases like Type 2 Diabetes (Liu et al., 2013)(Liu et al., 2013)(Liu et al., 2013)(Liu et al., 2013).

Collective epidemiological evidence points to the obesogenic potential of LMW-PAHs. However, the lack of mechanistic studies on the effects of individual LMW-PAH *in vitro* and *in vivo* leaves a gap in understanding their contribution to obesity and related pathologies. The intimate relationship between adipose tissues and the immune system warrants additional questions about the cellular targets of LMW-PAHs. Further investigation could unveil the impact of LMW-PAHs on adipogenic homeostasis and their ensuing impact on overall metabolic health.

### ***1.3.3 Biototoxicity of polycyclic aromatic hydrocarbons***

Many environmental pollutants are either adipogenic or have the potential to become adipogenic once metabolized in the body. PAHs are genotoxic, carcinogenic, and possibly adipogenic (Diggs et al., 2011; Pelkonen & Nebert, 1982; Schultz & Sinks, 2002). These fat-soluble compounds tend to accumulate in fatty tissues, such as the adipose and liver tissues (Kilanowicz et al., 1999; Melancon & Lech, 1978; Tarshis & Rattner, 1982). In recent years, significant progress has been made toward identifying targeted molecular pathways of PAH toxicity. Emerging studies provide evidence of biototoxicity that often afflict future generations of test subjects. Progeny of mice exposed to PAH mixtures during gestation exhibited increased adiposity and expression of canonical adipogenic genes (Z. Yan et al., 2014). There is an influx of reports describing the inflammatory consequences of PAH in a range of experimental models. Male mice exposed to



automobile exhaust presented increased responsiveness to LPS treatments, producing higher levels of peripheral IL-1 $\beta$  (Bolton et al., 2014). RAW 264.7 macrophages incubated with PAHs extracted from incense smoke produced significantly higher amounts of TNF- $\alpha$  and nitric oxide (Yang et al., 2017). Elevated expression of classical activation markers is seen in macrophages exposed to particulate-bound PAH mixtures (Fu et al., 2020)(Fu et al., 2020)(Fu et al., 2020)(Fu et al., 2020). Mice chronically exposed to air pollution saw significant reductions in thermogenic gene expression and, subsequently, BAT thermogenesis (Campolim et al., 2020)(Campolim et al., 2020)(Campolim et al., 2020)(Campolim et al., 2020). The well-studied HMW-PAH, Benzo(a)pyrene, has been evaluated across a multitude of *in vitro* and *in vivo* models, including human cell models. Acute and chronic benzo[a]pyrene treatments in mice result in increased body mass. Upon further examination, adipose tissues extracted from the treatment groups displayed decreased lipolysis via  $\beta$ AR antagonism (Irigaray et al., 2006). Many researchers are beginning to shift focus onto LMW-PAHs, which have been shown to have cardiotoxic, immunotoxic, and pro-inflammatory effects (Guo et al., 2021; Incardona et al., 2004; Marris et al., 2020; Rand et al., 2017).

Despite the wealth of literature available in the field, there is a lack of mechanistic studies focused on how individual LMW-PAHs contribute to metabolic disorders. However, these compounds and their metabolic products have been extensively characterized (Bojes & Pope, 2007; Wilson et al., 1996). It is a natural idea to begin investigations with the structurally smallest PAH, naphthalene. Acute naphthalene exposure is cytotoxic and genotoxic to macrophages, encouraging the production of damaging free radicals (Bagchi et al., 1998). There is also evidence of naphthalene causing respiratory distress through inflammatory mechanisms (Serikov et al., 2005; van Winkle

et al., 2002). Findings from previous collaborations now provide evidence of 2-NAP accelerating preadipocyte maturation and activating immunological responses *in vitro*. Further examination revealed multiple effects of chronic 2-NAP exposure during the early stages of differentiation. Chronic 2-NAP exposure leads to the activation of key adipogenic transcriptional regulators, modulates antimicrobial peptide production, and disrupts insulin signaling pathways (Wang, 2015). During early differentiation, 2-NAP stimulates C/EBP $\beta$ , IRS2, and SREBP1c expression and upregulates C/EBP $\alpha$ , PPAR $\gamma$ , and aP2 expression during terminal differentiation. 2-NAP also elevates the expression of the antimicrobial peptide, Camp. This work demonstrated the adipogenic and inflammatory potential of 2-NAP (Wang, 2015). Yet, even with this knowledge, there is still a great deal of progress to be made in this field. The specific mechanisms by which 2-NAP influences the development of obesity and related metabolic disorders need further evaluation.

#### **1.4 *Daphnia magna*: An emerging model for metabolic disorders**

*D. magna*, commonly referred to as the water flea, is a micro-crustacean native to freshwater environments. Due to their extreme sensitivity to environmental changes, daphnia are considered indicator species and a popular model in toxicological studies. The use of daphnia in scientific research dates to the early 1900s (MacArthur & Baillie, 1926; Stross, 1971). Since then, researchers have characterized various physical and biochemical processes, mapped the genome of commonly used laboratory species, and established basic protocols that allow for indefinite maintenance of individual clones in the laboratory setting (Biesinger et al., 1987; Colbourne et al., 2011; Dudycha & Tessier, 1999; Shaw et al., 2008). *D. magna* as a biological *in vivo* model offers several advantages when examining environmental influences on lipid dynamics. Compared to

other sequenced invertebrate genomes, daphnia share the highest genomic homology with humans (Shaw et al., 2008). Daphnia also possess regenerative properties, including cell proliferation and renewal (Benzie, 2005). Many high energetic processes like molting, growth, and reproduction rely on lipid homeostasis. Disruptions in lipid or energy homeostasis pathways can be linked to a defined phenotype (Soetaert et al., 2007; Zou & Fingerman, 1997). Logistically, daphnia are a cost-efficient, and cultures are easy to maintain in a laboratory setting. Under favorable conditions, daphnia prefer asexual reproduction, producing genetically identical clones, that reduce misinterpretation of data due to genetic confounders. The daphnia's translucent exoskeleton makes it possible to view anatomical structures. These traits, combined with short reproductive life cycles, make *D. magna* an attractive model system for the disentanglement of genetic and environmental cross-talks involved in lipid metabolism.

As well-established ecotoxicological models, daphnia serve as an excellent model system for investigating potential targets of environmental toxins. Since the characterization of the *D. pulex* and *D. magna* genome, the use of daphnia in other research settings have emerged. Lipids are the primary source of energy for metabolic maintenance and fecundity in daphnia (Arrese & Soulages, 2010). The disturbance of lipid production and trafficking disrupts high energy processes necessary for successful embryo development and molting. Oocyte development induce transportation of triglycerides from adipocytes in the hemocoel to the ovary (Dudycha & Tessier, 1999). Additionally, chemicals that alter storage lipids also impair normal molting patterns (R. Jordão et al., 2016; R. G. Jordão et al., 2016). These lipid-altering events induce phenotypes easily identified during metabolic disturbances (Goulden & Place, 1993; Wacker & Martin-Creuzburg, 2007). Lipids are the building blocks of cellular biology, fuel all energetic processes of the cell,

and are essential in cellular communication and protein trafficking. Disruption of lipid homeostasis often leads to the development of metabolic disorders, including but not limited to; obesity, diabetes, inflammatory disorders, and coronary disorders. Environmental exposures often contribute to the development of such disorders by targeting lipid-associated pathways. Master transcriptional regulators, like the peroxisome proliferator-activated receptors, are key regulators in lipid maintenance. Researchers have been successful in identifying environmental pollutants associated with these metabolic disorders. However, the complexities of lipid regulation in higher-level organisms add difficulties to identifying the specific targets of these pollutants.

Adipocyte regulation and lipid metabolism are the two most prominent areas of interest when studying the effects of obesogens. A research group from Spain has developed the framework for using the daphnia model to explore the cellular mechanisms of obesogens. Recently, (R. Jordão et al., 2015; R. G. Jordão et al., 2016) examined the effect of other known environmental obesogens (Tributyltin (TBT), BPA, and DEHP, among others) using *D. magna* as their animal model.

As noted earlier, 2-NAP has been shown to accelerate the maturation process from fibroblast into adipocytes. Transcription factors *C/EBP $\alpha$*  and *PPAR $\gamma$*  are master regulators of mammalian adipocyte differentiation. Mouse pre-adipocytes cultured *in vitro* with 2-NAP differentiate into mature adipocytes earlier and display higher amounts of intracellular lipids with increased *C/EBP $\alpha$*  and *Ppar $\gamma$*  mRNA expression (Wang, 2015). RXR is another regulator of adipogenesis and a known heterodimeric partner of *PPAR $\gamma$* . Transcription factors such as *PPAR $\gamma$*  and RXR are typically the main targets of most obesogens (Metzger et al., 2005). While daphnia do not express *PPAR $\gamma$* , they do express an RXR homolog called ultraspiracle (Iguchi & Yoshinao, 2008).

Daphnia RXR dimerizes with ecdysteroid receptors to activate the transcription of adipogenic genes. Agonists of ecdysteroids have been reported to have effects on survival, molt, growth and/or reproduction (Taubert et al., 2011). Jordao et al. did, indeed, demonstrate TBT's ability to increase lipid accumulation and upregulate transcription of RXR and various other associated proteins. They also showed that TBT was able to activate the RXR-EcR complex. They were able to implicate *D. magna* as a potential model to evaluate the disruptive effects of obesogens such as 2-NAP and other environmental pollutants.

Establishing *D. magna* as an *in vivo* model for mechanistic and genetic studies evaluating the effects of obesogenic compounds in; lipid metabolism, immune function, and adipocyte development could ultimately lead to a cost-effective *in vivo* system that would allow researchers to identify, target, and assess endocrine disruptors quickly and efficiently.

## **1.5 Overview of research aims**

Polycyclic aromatic hydrocarbons are ubiquitous environmental pollutants that are known to be adipogenic, carcinogenic, and genotoxic (Pelkonen & Nebert, 1982; Schultz & Sinks, 2002; Z. Yan et al., 2014). PAHs are fat-soluble and accumulate in fatty tissues, such as adipose and liver tissues (Kilanowicz et al., 1999; Melancon & Lech, 1978; Tarshis & Rattner, 1982). Urinary naphthalene metabolites act as biomarkers of PAH exposure. Naphthalene metabolite concentrations are positively correlated with the prevalence of childhood obesity (Scinicariello & Buser, 2014). Naphthalene metabolite, 2-NAP, can accelerate preadipocyte maturation and activate Immunological responses *in vitro* (Wang, 2015). However, specific mechanisms by which

naphthalene, structurally the simplest PAH, and its metabolites influence the development of obesity and related metabolic disorders remain unclear.

Identifying potential chemical disruptors as obesogens allows us to fully comprehend the etiology and design management of diseases such as obesity, insulin resistance, type 2 diabetes, and metabolic syndrome. To explore this idea, we aimed to determine the molecular mechanisms by which naphthalene, and its metabolites, influence the development of obesity and related metabolic disorders. The long-term goal of this project is to identify cellular pathways targeted by the LMW-PAH, 2-NAP. The effects of 2-NAP will be demonstrated both *in vivo* and *in vitro*. **The overarching hypothesis is that chronic exposure to LMW-PAHs perturbs cellular mechanisms that govern obesogenic phenotypes and associated metabolic activity by promoting cellular lipid accumulation and inflammation.**

The rationale driving this proposal stems from the background and preliminary data that shows a strong relationship between chronic low-grade 2-NAP exposure and disruptions in lipid homeostasis, production of inflammatory cytokines, and increased incidences of metabolic diseases. We employed two *in vitro* cellular models representing white adipocytes and brown adipocytes alongside *D. magna* – for *in vivo* studies – to investigate the role of individual LMW-PAHs on adipocyte function and lipid metabolism. White adipocytes and macrophages were used to determine the impact of LMW-PAH exposure on inflammation. We hope to use the results generated from the proposed studies to identify the role of 2-NAP in metabolic homeostasis, obesity, and to help improve future therapeutic strategies for related disorders.

***1.5.1 Specific Aim 1: The effects of low-molecular-weight polycyclic aromatic hydrocarbons on lipid accumulation and metabolic inflammation in vitro.***

***1.5.1.1 Aim 1.1: Define the role of LMW-PAHs in adipocyte function and lipid metabolism.***

Adipose tissue serves as a mediator between immunological and metabolic functions. Dysregulation of adipose tissue often leads to low-grade chronic inflammation and, eventually, metabolic distress. We will employ 3T3-L1 and BAT-1 cell lines to explore the effects of 2-NAP on lipid metabolism in vertebrate systems. We are particularly interested in 2-NAP's effects on lipid accumulation and the regulation of adipogenic gene expression during differentiation. This study will also monitor 2-NAP's effect on thermogenic gene expression in brown. We will examine both chronic and acute outcomes of adipocytes exposed to the LMW-PAH. This aim is divided into the following sub-aims: 1.11) PAH regulation of adipogenesis white adipocytes. 1.12) PAH's role in metabolic function across differentiation in brown adipocytes.

***1.5.1.2 Aim 1.2: Determine the impact of LMW-PAH exposures on inflammation in vitro.***

In addition to inducing differentiation, 2-NAP activates inflammatory responses in pre-adipocyte cells (Wang, 2015)(Wang, 2015)(Wang, 2015)(Wang, 2015). We must also consider the role of inflammation in these metabolic dysfunctions. Evidence of cross-regulation between adipocytes and immune cells warrants the exploration of this aim. Macrophage function is critical to developing, progressing, and preventing many metabolic disorders (Ferrante, 2013; Remmerie & Scott, 2018)(Ferrante, 2013; Remmerie & Scott, 2018)(Ferrante, 2013; Remmerie & Scott, 2018)(Ferrante, 2013; Remmerie & Scott, 2018). We will examine the transcriptional regulation of inflammatory genes in white and brown adipocytes. In addition to the previously described

adipocyte models, we will employ the murine monocyte/macrophage-like *in vitro* model system using RAW 264.7 cells to determine the effects of LMW-PAHs on metabolic inflammation. This aim will investigate mechanisms targeted by LMW-PAHs that modulate cytokine production and plasticity of macrophages and adipocytes. This aim was further subdivided as presented: 1.21) Characterize PAH-mediated macrophage inflammation. 1.22) Characterize acute PAH-mediated responses in mature adipocytes.

***1.5.2 Specific Aim 2: Explore the in vivo effects of chronic 2-naphthol exposure in the invertebrate Daphnia magna.***

Life history events in *D. magna*, such as molting, development, fecundity, and lifespan, depend on lipid homeostasis. In turn, an alteration in lipid content will indirectly influence life-history events (Barata & Baird, 1998; Wacker & Martin-Creuzburg, 2007). Typically used in toxicological studies, *D. magna* are emerging as an attractive model for studying the effects of environmental contaminants on lipid homeostasis. The nuclear receptors PPAR $\gamma$  and RXR form heterodimers that regulate a battery of adipogenic genes involved in metabolic homeostasis and inflammation. Jordao et al. (2015) determined that exposure of daphnia to the obesogen, TBT, increased levels of RXR mRNA (R. Jordão et al., 2015). Previous data revealed that 2-NAP increased the expression of *Ppar $\gamma$*  and other crucial genes involved in adipocyte development (Wang, 2015). We will utilize the *D. magna* model to explore the following sub-aims: 2.1) Investigate the consequences of 2-NAP on individual and population life-history traits in *D. magna in vivo*. Furthermore, 2.2) Examine the influence of 2-NAP on lipid accumulation *in vivo*.



## **2 SPECIFIC AIM 1: THE EFFECTS OF LOW-MOLECULAR-WEIGHT POLYCYCLIC AROMATIC HYDROCARBONS ON LIPID ACCUMULATION AND METABOLIC INFLAMMATION *IN VITRO*.**

### **2.1 Abstract**

2-naphthol, a metabolite of naphthalene, is a low-molecular-weight (LMW) polycyclic aromatic hydrocarbon (PAH) and air pollutant associated with childhood obesity. There has been a recent emergence of studies on the consequences of PAHs on human health. Current epidemiological reports suggest LMW-PAHs may contribute to a significant proportion of the overall burden of obesity in children, yet most studies focus on high-molecular-weight PAHs. This study explores 2-naphthol's impact on obesity and obesity-associated metabolic disorders. To investigate 2-naphthol's effect on lipid metabolism and inflammation, we employed 3T3-L1 and BAT1 cell lines to model white and brown adipocytes, respectively, alongside a murine macrophage cell line (RAW264.7). Cells were challenged with either acute or chronic 2-naphthol treatments. Differentiated adipocytes were assessed for changes in gene expression in adipogenesis, lipid accumulation, and isoproterenol-stimulated lipolysis and thermogenesis. Additionally, LPS-induced inflammation was assessed in both adipocytes and macrophages. Results show that 2-naphthol increased the expression of key adipogenic and lipogenic genes while decreasing lipolytic gene expression in both chronically treated adipocyte cell lines. In consistence, an increase in lipid accumulation was demonstrated in BODIPY and Oil Red O-stained adipocytes. Additionally, adipocytes and macrophages chronically exposed to 2-naphthol showed up-regulated mRNA expression of major inflammatory cytokines (eg, *Tnfa*, *Il-1 $\beta$* , *Il-6*). In summary, chronic exposure to 2-naphthol stimulates lipid accumulation in adipocytes and inflammation in adipocytes and

macrophages. These findings support previous research that demonstrates 2-naphthol has obesogenic potential.

## **2.2 Introduction**

Because we cannot solely rely on correlative studies as sole predictors of illness, exposure-based mechanistic studies are needed to establish causation. Limited research alongside 2-NAP's biochemical properties hints toward its potential to influence metabolic dysfunction through immunomodulatory mechanisms. For example, studies have shown that mice exposed to a PAH mixture in utero weighed more and had increased adiposity compared to control mice including the increased expression of adipogenic and lipogenic genes in adipose tissues (Yan et al., 2014). HMW-PAHs, benzo(a)pyrene and 1-nitropyrene, increase the expression of the proinflammatory cytokines IL-8, IFN $\gamma$ , and IL-1 $\beta$  in lung epithelial cells (Pei et al., 2002) and splenocytes (Vandebriel et al., 1998). Individual HMW-PAHs can also up-regulate CCL1 expression (Ndiaye et al., 2006) alongside mRNA and protein TNF $\alpha$  levels in cultured human macrophage cells (Lecureur et al., 2005). Additionally, activated macrophages treated with individual PAHs showed increased antigen presentation (Myers et al., 1987) and IL-1 production proportionate to lipopolysaccharide stimulation (Lyte & Bick, 1986). Evidence of individual PAHs activating inflammatory pathways combined with increased associations between LMW-PAHs and obesity warrants further investigation into the impact of 2-NAP on inflammation and lipid metabolism.

Thus, this research aimed to 1) Investigate the role of the LMW-PAH 2-NAP on adipocyte function and lipid metabolism. 2) Determine the impact of 2-NAP exposure on inflammation. The findings

of this study may lead to further exploration into the cellular pathways targeted by LMW-PAHs, increasing our understanding of how individual PAHs contribute to metabolic dysfunction.

## **2.3 Materials and Methods**

### ***2.3.1 Cell culture***

RAW 264.7 murine macrophages (ATCC, Manassas, VA) were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen 11965-092) containing 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S). Cells were incubated at 37° C in an atmosphere of 5% CO<sub>2</sub>.

3T3-L1 murine preadipocytes (ATCC, Manassas, VA) were cultured in an expansion medium (EM) containing DMEM supplemented with 10% calf serum (CS) and 1% penicillin/streptomycin (P/S) and incubated as described above. Once preadipocytes reached confluency, cells were cultured for 48 hours in differentiation media (DM), containing the growth media (DMEM + 10% FBS + 1% P/S) supplemented with 400 nM insulin, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX), and 1 μM dexamethasone (DEX). Cell culture media was then replaced with maintenance medium (MM), containing the growth media (DMEM + 10% FBS + 1% P/S) supplemented with 400 nM insulin for 2 additional days following differentiation. On day 5, culture media was replaced with MM every 2 days until 3T3-L1 cells reached maturity on day 8.

BAT1 murine preadipocyte (a generous gift from Dr Patrick Seale, University of Pennsylvania) cultures were grown in a BAT1 expansion medium (BEM) containing DMEM/Nutrient Mixture F-12 (DMEM/F12, Invitrogen, 11330-032) supplemented with 10% FBS and 1% P/S for 2 days

and incubated as described above. Once cells reached 90% confluency, the BEM was replaced with a BAT1 differentiation media (BDM), containing BEM supplemented with 20 nM insulin, 0.5 mM IBMX, 1  $\mu$ M DEX, 125  $\mu$ M indomethacin, and 1 nM triiodothyronine (T3) for 2 days. After the 2-day differentiation period, the media was replaced with BAT1 maintenance medium (BMM), containing BEM supplemented with 20 nM insulin and 1 nM T3, every 2 days until day 8.

### ***2.3.2 RNA extraction and RT-PCR analysis***

Cellular RNA was extracted from cultures using the Tri Reagent kit (Molecular Research Center, RT 111), as described in the manufacturer's protocol for cells grown in monolayer. ABI Universal PCR Master Mix and TaqMan primer and probe pair reagents were acquired from Applied Biosystems. Gene expression was evaluated using quantitative RT-PCR using Applied Biosystems QuantStudio 3 real-time PCR system (ThermoFisher Scientific). Target mRNA expression levels were normalized to the expression of the housekeeping gene, cyclophilin, relative to controls. We used cyclophilin primer and probe sequences: 5'-GGTGGAGAGCACCAAGACAGA-3'(forward), 5'-GCCGGAGTCGACAATGATG-3'(reverse), and 5'-TCCTTCAGTGGCTTGTCCTCCGGCT-3'(probe). All other gene expression primers and probes were purchased from Applied Biosystems. Corresponding cycle threshold (Ct) values were measured, and relative mRNA level was determined using the  $2(-\Delta\Delta Ct)$  method.

### ***2.3.3 Enzyme-linked immunosorbent assays (ELISA)***

RAW 264.7 macrophages were cultured as described above. TNF- $\alpha$  protein production in the cell culture medium was measured using the Mouse TNF-alpha Quantikine ELISA Kit (R&D Systems™, MTA00B) according to the manufacturer's protocol.

### ***2.3.4 Staining and visualization of neutral lipids***

Oil Red O (ORO, Sigma-Aldrich, O9755) staining was employed to visualize lipid accumulation during 3T3-L1 adipogenesis. In preparation for staining, differentiated 3T3-L1 cells were cultured in 6-well plates, washed with PBS, and fixed with 10% formalin overnight. After fixation, cells were washed twice with MilliQ ddH<sub>2</sub>O and incubated in a 0.5% ORO solution prepared in isopropanol for 10 minutes at 37°C. Plates were rinsed 4 times with ddH<sub>2</sub>O, air-dried, and photographed directly.

To visualize intracellular lipid droplets, triglycerides were labeled with 4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene (BODIPY 493/503, Thermo Fisher Scientific, D3922) as previously described (Qiu & Simon, 2016). 3T3-L1 adipocytes were seeded on 12mm collagen-treated glass coverslips at a density of  $5 \times 10^4$  cells/well in 24-well plates. After differentiation and treatment, as indicated under the figures, cells were fixed in 4% paraformaldehyde for 30 minutes at room temperature. Cells were then incubated in a 2  $\mu$ M BODIPY solution in the dark for 15 minutes at 37°C and washed twice with PBS to remove the residual stain. Coverslips were mounted onto glass slides with Prolong Diamond Antifade Mountant (Thermo Fisher Scientific). Slides were cured overnight in the mounting solution, imaged, and immediately stored at 4°C. Images of lipid droplets were captured at excitation/emission wavelengths 493/503 nm using an Olympus DP73 photomicroscope and CellSens software (Olympus, Waltham, MA).

### 2.3.5 Statistical analysis

Reported data are expressed as mean  $\pm$  SEM. Differences between treatment groups were analyzed for statistical significance by ANOVA with Fischer's probable least-squares difference post hoc. Significantly different group means ( $p < 0.05$ , indicated by an asterisk \*) were calculated using GraphPad Prism version 9 (GraphPad Software, La Jolla, California, USA, [www.graphpad.com](http://www.graphpad.com)).

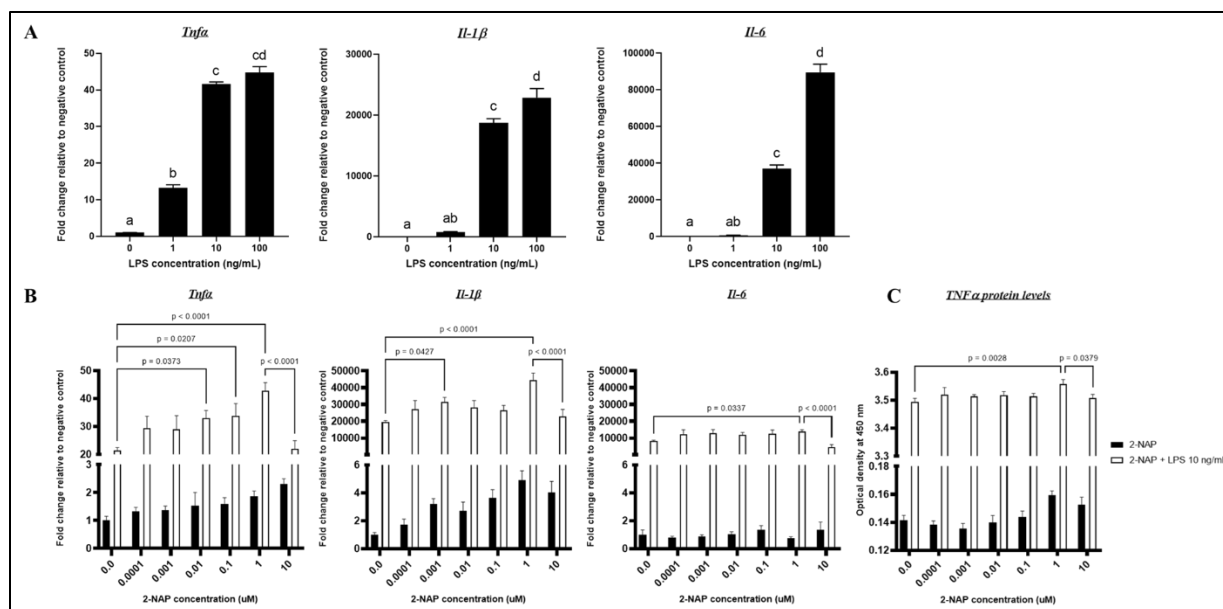
## 2.4 Results

### 2.4.1 *In vitro* effects of varying LPS concentrations on RAW264.7 macrophages

Lipopolysaccharide (LPS) is commonly used to stimulate proinflammatory activation of macrophages *in vitro* (Baris et al., 2021). Proinflammatory cytokines *Tnfa*, *Il-1 $\beta$* , and *Il-6*, are produced by macrophages and adipocytes, the primary cells involved in obesity and metabolic disorders. Given that these cytokines are elevated under obese conditions and promote chronic low-grade inflammation in adipose tissue (Kim & Bajaj, 2014), we measured *Tnfa*, *Il-1 $\beta$* , and *Il-6* mRNA transcript levels to assess the consequences of LPS stimulation in macrophages. RAW 264.7 cells were stimulated *in vitro* across a range of LPS concentrations, from 1 ng/ml to 100 ng/ml, to establish an optimal dose of LPS. As shown in Fig. 2.2.1A, *Tnfa* and *Il-1 $\beta$*  mRNA levels displayed dose-dependent responsiveness upon LPS stimulation that peaked at a concentration of 10 ng/ml, whereas the expression of *Il-6* was significantly increased across all concentrations (up to the highest LPS dose — 100 ng/ml). Given that PAHs can also activate macrophages and drive proinflammatory responses (Fu et al., 2020), the 10 ng/ml LPS concentration was chosen to prevent saturating the proinflammatory responses of RAW 264.7 cells. Thus, the LPS dosage of 10 ng/ml was used for subsequent experiments throughout this study.

#### ***2.4.2 Acute 2-NAP treatment enhances inflammatory responses in RAW264.7 macrophages***

Macrophage activation is an essential feature of metabolic inflammation. To assess the effect of 2-NAP on macrophage inflammation, we measured cytokine gene expression in RAW 264.7 cells across a wide range of 2-NAP concentrations (six 10-fold concentrations, 0.0001  $\mu\text{M}$  – 10  $\mu\text{M}$ ) in the absence or presence of 10ng/ml LPS for 4 hours. Results showed that acute 4-hour 2-NAP treatment in RAW 264.7 macrophages had apparent effects on basal *Tnf $\alpha$* , *Il-1 $\beta$* , and *Il-6* expression (Fig. 2.1B). LPS significantly stimulated expression of these genes in RAW 264.7 macrophages; 2-NAP further enhanced LPS' effect, with the largest expression enhancement occurring at a 2-NAP concentration of 1  $\mu\text{M}$  (Fig. 2.1B). Furthermore, ELISA analysis of supernatants of LPS-activated RAW 264.7 cells treated across the same 2-NAP range revealed that 2-NAP increased basal TNF $\alpha$  levels and significantly enhanced LPS-stimulated TNF $\alpha$  secretion at a 2-NAP concentration of 1 $\mu\text{M}$  level (Fig. 2.1C). We also found that 10 $\mu\text{M}$  2-NAP treatment reduced *Tnf $\alpha$* , *Il- $\beta$* , and *Il-6* expression, as well as TNF $\alpha$  secretion as compared to that of 1 $\mu\text{M}$  2-NAP (Fig 2.1B-C); however, this may be due to 2-NAP's toxicity at high doses as it was found that 10 $\mu\text{M}$  2-NAP reduced adult survival rate in *Daphnia magna*, a model system commonly used in toxicological studies, during chronic treatment (Table 3.1). Thus, 1 $\mu\text{M}$  2-NAP was used in the subsequent studies.



**Figure 2.1 Acute 2-NAP treatment of RAW 264.7 macrophages up-regulates proinflammatory cytokine expression.**

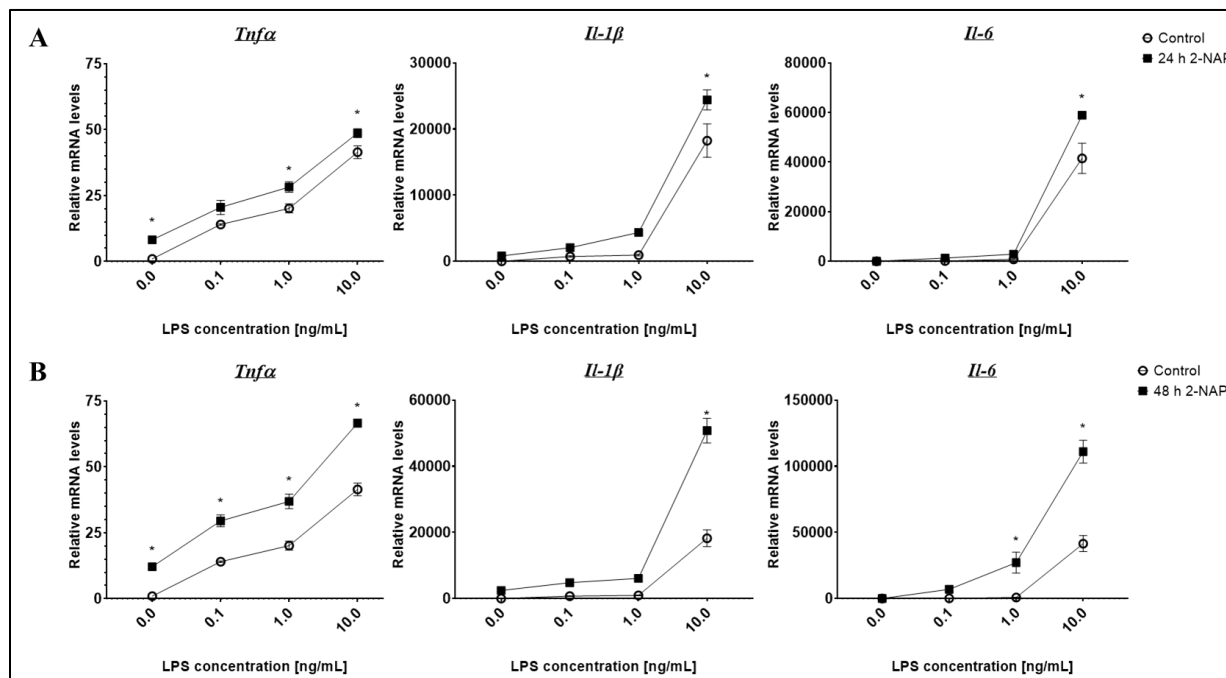
(A) The effect of LPS on proinflammatory gene expression in RAW 264.7 macrophages. Cells were stimulated with LPS for 4 h. Following stimulation, mRNA was extracted and assayed for relative gene expression of *Tnfα*, *IL-1β*, and *IL-6* by RT-PCR. The values shown represent the mean  $\pm$  SEM of replicate samples from one experiment ( $n = 4$ ). Groups labeled with different letters (a, b, c, and d) are statistically different from each other. (B) Macrophages were either treated with 2-NAP alone—six 10-fold concentrations (10 pM–10  $\mu$ M concentrations) or stimulated with LPS (10 ng/mL) alongside 2-NAP treatment. LPS was used to mimic chronic inflammatory (M1-like) conditions. Solvent control—DMEM + 0.01% ethanol (EtOH); LPS control—DMEM + LPS 10 ng/mL. Upon experimental termination, mRNA was extracted and relative gene expression of *Tnfα*, *IL-1β*, and *IL-6* mRNA was then determined by RT-PCR ( $n = 4$ ). The values shown represent the mean  $\pm$  SEM. A  $p$ -value of  $p < 0.05$  indicates statistical significance between two groups. (C) Protein expression of TNF $\alpha$  extracted from cellular supernatants ( $n = 4$ ). The optical density of each sample represents readings of TNF $\alpha$  levels secreted into the medium by ELISA assay and was measured using a microplate reader set to 450 nm. Wavelength correction was set to 570 nm. The values shown represent the mean  $\pm$  SEM. A  $p$ -value of  $p < 0.05$  indicates statistical significance between two groups.

### 2.4.3 Chronic 2-naphthol exposure increases the expression of inflammatory genes in macrophages

Cytokine mRNA expression was measured in RAW 264.7 macrophages cultured under chronic 2-NAP exposure conditions (Fig. 2.2). RAW 264.7 macrophages were treated with 1 $\mu$ M 2-NAP for



either 24 or 48 hours, then challenged with various concentrations of LPS for 4 hours. We found that chronic 2-NAP treatment for 24 (Fig. 2.2A) or 48 (Fig. 2.2B) hours significantly increased basal *Tnfa* expression and significantly enhanced LPS-induced inflammatory gene expression, including *Tnfa*, *Il-1 $\beta$* , and *Il-6* (Fig. 2.2).

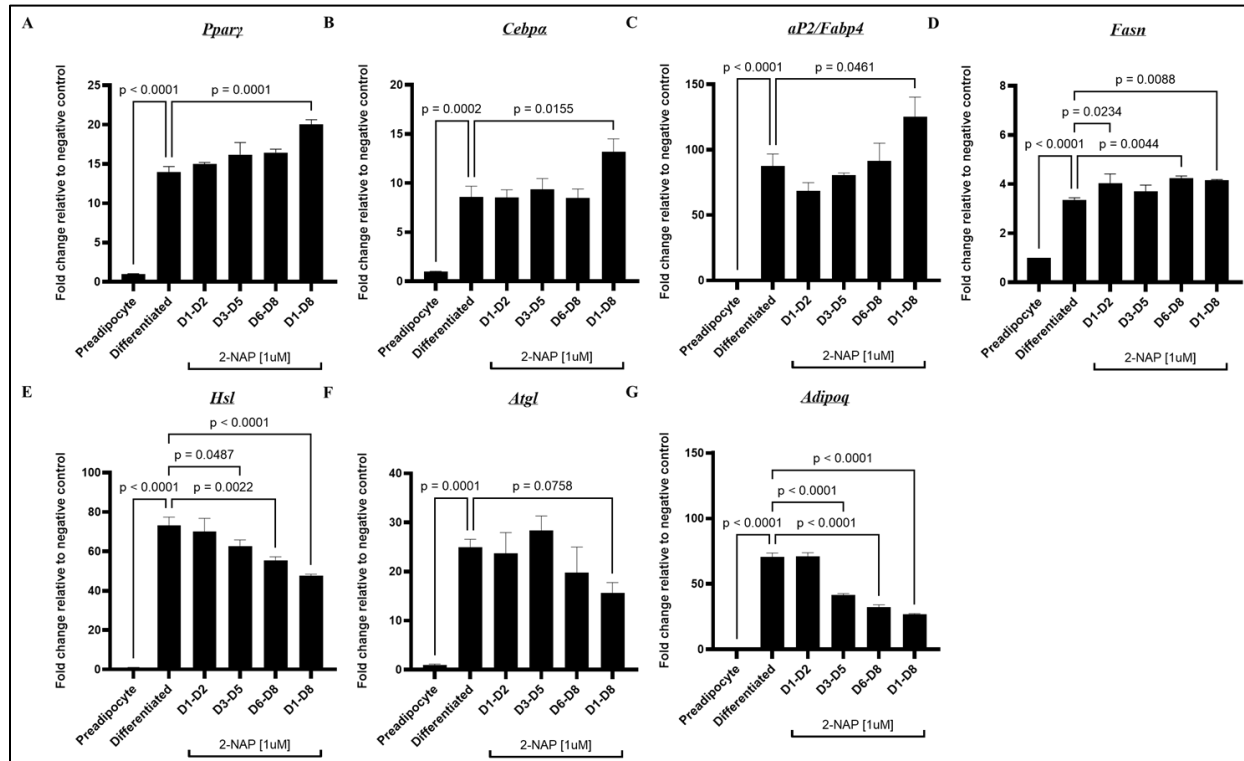


**Figure 2.2 Chronic 2-NAP treatment of RAW 264.7 macrophages up-regulates mRNA expression of proinflammatory cytokines.**

Chronic 2-NAP treatment of RAW 264.7 macrophages upregulates mRNA expression of proinflammatory cytokines. (A,B) Relative mRNA expression levels of proinflammatory markers (*Tnfa*, *Il-1 $\beta$* , and *Il-6*) in cells treated with 2-NAP (1  $\mu$ M) for 24 (A) and 48 (B) hours. RAW 264.7 macrophages were first treated with or without 1  $\mu$ M 2-NAP for 24 (A) or 48 (B) hours, and then challenged with different concentrations of LPS as indicated for 4 h. Upon experimental termination, mRNA was extracted and relative gene expression of *Tnfa*, *Il-1 $\beta$* , and *Il-6* mRNA was then determined by RT-PCR (n = 4). The values shown represent the mean  $\pm$  SEM. \* Denotes  $p < 0.05$  when compared with controls.

#### ***2.4.4 Chronic 2-naphthol treatment up-regulates an adipogenic and lipogenic program gene expression in 3T3-L1 cells***

Next, we sought to investigate the effects of 2-NAP on adipogenesis and lipid metabolism. 3T3-L1 murine preadipocytes were induced to differentiate with a differentiation medium and were concurrently treated with either PBS or 2-NAP on day 1–2, 3–5, 6–8, or 1-8 of differentiation. Cells from all treatment groups were harvested at day 8 of differentiation. Adipocytes exposed to 2-NAP during differentiation show an overall increase in adipogenesis (Fig. 2.3). More specifically, chronic 2-NAP treatment, especially during the entire 1-8 days of differentiation, up-regulated 3T3-L1 adipogenesis, as shown by increased mRNA expression of adipogenic markers such as peroxisome proliferator activated receptor gamma (*Pparγ*), CCAAT enhancer binding protein alpha (*Cebpa*), and adipocyte protein 2/fatty acid binding protein 4 (*aP2/Fabp4*) (Fig.3). Additionally, chronic 2-NAP treatment from days 1-8 increased the expression of a key lipogenic enzyme, fatty acid synthase (*Fasn*), while coordinately downregulating genes involved in lipolysis, including the significant downregulation of hormone sensitive lipase (*Hsl*) and the tendency of downregulation of patatin-like phospholipase domain containing 2/adipocyte triglyceride lipase (*Pnpla2/Atgl*) (Fig. 2.3). Moreover, chronic 2-NAP treatment during differentiation significantly decreased the adipokine adiponectin (*Adipoq*) expression (Fig. 2.3). In 3T3-L1 cells treated with 2-NAP during the differentiation days 6-8, there was also an increase in *Fasn* expression and a coordinated decrease in *Hsl* expression (Fig. 2.3). Thus, our data suggest that chronic 2-NAP treatment in adipocytes may promote an adipogenic and lipogenic program by coordinated regulation of gene expression involved in adipogenesis, lipogenesis, and lipolysis.



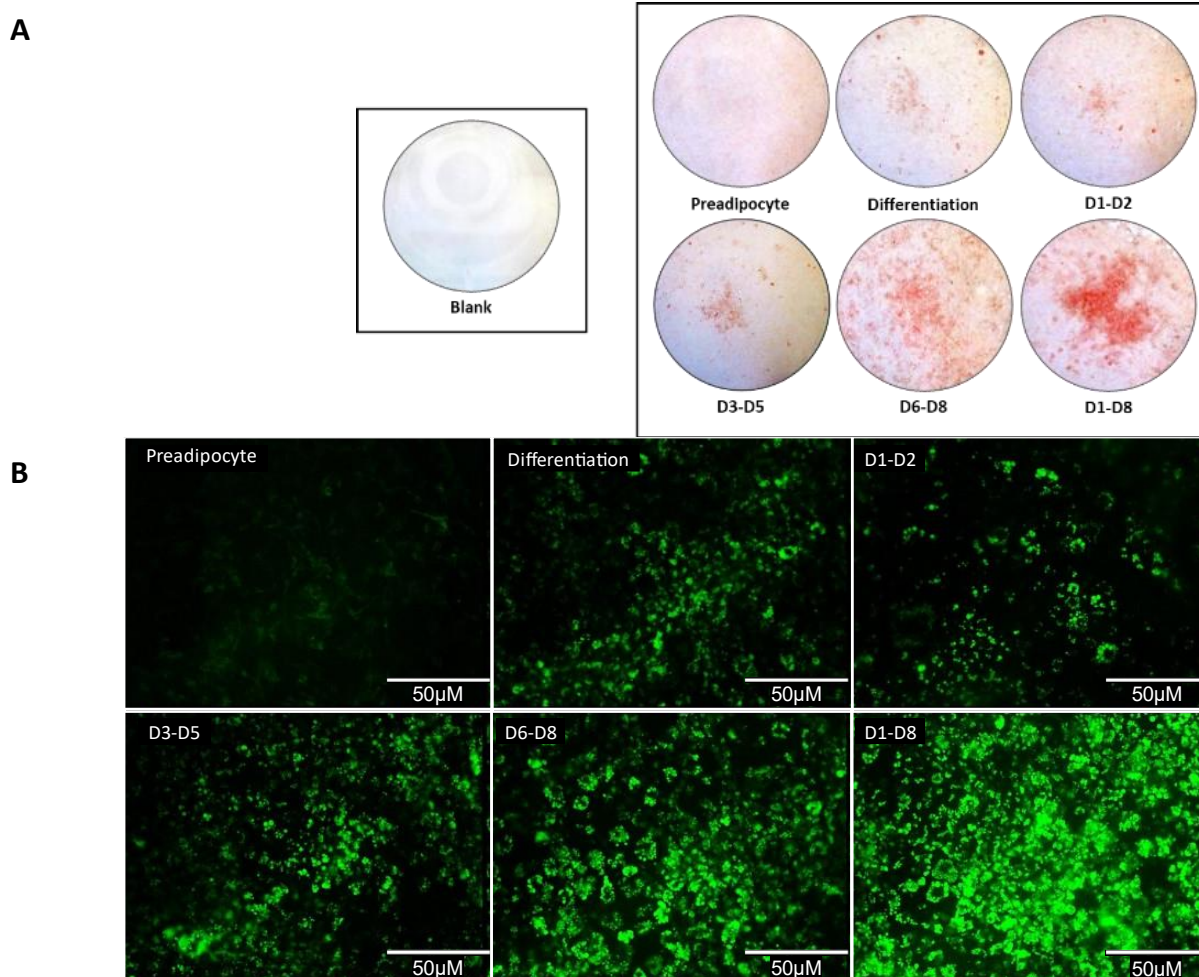
**Figure 2.3 Chronic 2-naphthol treatment up-regulates adipogenic, lipogenic, and metabolic gene expression in 3T3-L1 adipocytes.**

Relative mRNA expression of genes encoding for adipogenic transcription factors *Pparγ* (A) and *Cebpa* (B); adipocyte specific marker *ap2/Fabp4* (C), lipogenic and lipolytic markers *Fasn* (D); *Hsl* (E) and *Atgl* (F); and the adipokine *Adipoq* (G) was measured in preadipocytes and differentiated 3T3-L1 adipocytes treated with 1μM 2-NAP at different differentiation stages as indicated. Upon experimental termination, mRNA was extracted, and relative gene expression was then determined by RT-PCR (n = 4). The values shown represent the mean ± SEM. A *p*-value of *p* < 0.05 indicates statistical significance between two groups.

#### 2.4.5 Qualitative analysis shows an increase in lipid accumulation in 2-NAP treated 3T3-L1 adipocytes.

We then examined lipid accumulation in 3T3-L1 adipocytes treated with 2-NAP by either oil red O (ORO) or BODIPY staining. 3T3-L1 cells were photographed directly for a whole-well view (Fig. 2.4A) or at 40x magnification for a detailed microscopic view (Fig. 2.4B). Consistent with results in Fig. 2.3, both ORO and BODIPY staining revealed increased lipid accumulation in

adipocytes under chronic 2-NAP treatment from days 1-8 and 6-8 (Fig. 2.4). Adipocytes treated during the early and middle stages of differentiation (days 1-2 and 3-5) did not show a measurable increase in lipid accumulation compared to the differentiation control (Fig. 2.4).

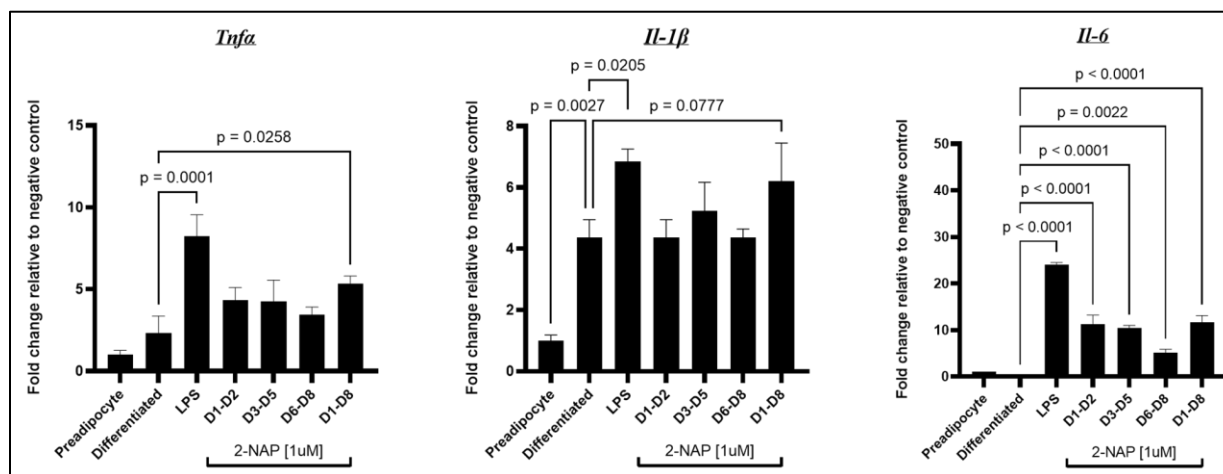


**Figure 2.4 Phenotypic analysis of 2-naphthol's effect on adipocyte differentiation and lipid accumulation by oil red O and BODIPY staining.**

Upon experimental termination, 3T3-L1 cells were fixed, stained, and photographed either directly (whole-well view) or at x40 magnification (microscopic view). **(A)** Whole-well imaging comparing ORO (red) stained 3T3-L1 preadipocytes differentiated under chronic 2-NAP treatments versus their controls. **(B)** Confocal microscopic images of BODIPY 493/503 (green) stained neutral lipid content in 3T3-L1 cells under chronic 2-NAP treatment. The scale bar spans 50 µM.

### 2.4.6 Chronic 2-NAP exposure increases the expression of inflammatory genes in 3T3-L1 cells

To determine whether chronic 2-NAP treatment in 3T3-L1 cells contributes to adipocyte inflammation, we treated 3T3-L1 cells with 1  $\mu$ M 2-NAP at specific points during differentiation, from differentiation days 1-2, 3-5, 6-8, and 1-8. Cells were harvested at differentiation day 8 for gene expression analysis. For the positive control LPS treatment, 3T3-L1 cells were differentiated without 2-NAP treatment and treated with 10ng/ml LPS for 4 hours on differentiation day 8 before harvesting the cells. As expected, 3T3-L1 adipocytes treated with LPS significantly induced inflammatory gene expression, including *Tnfa*, *Il-1 $\beta$* , and *Il-6* (Fig. 2.5). Interestingly, 3T3-L1 adipocytes chronically exposed to 2-NAP also showed a significant increase in mRNA expression of proinflammatory cytokine markers *Tnfa* and *Il-6* and a tendency for increased *Il-1 $\beta$*  expression (Fig. 2.5).



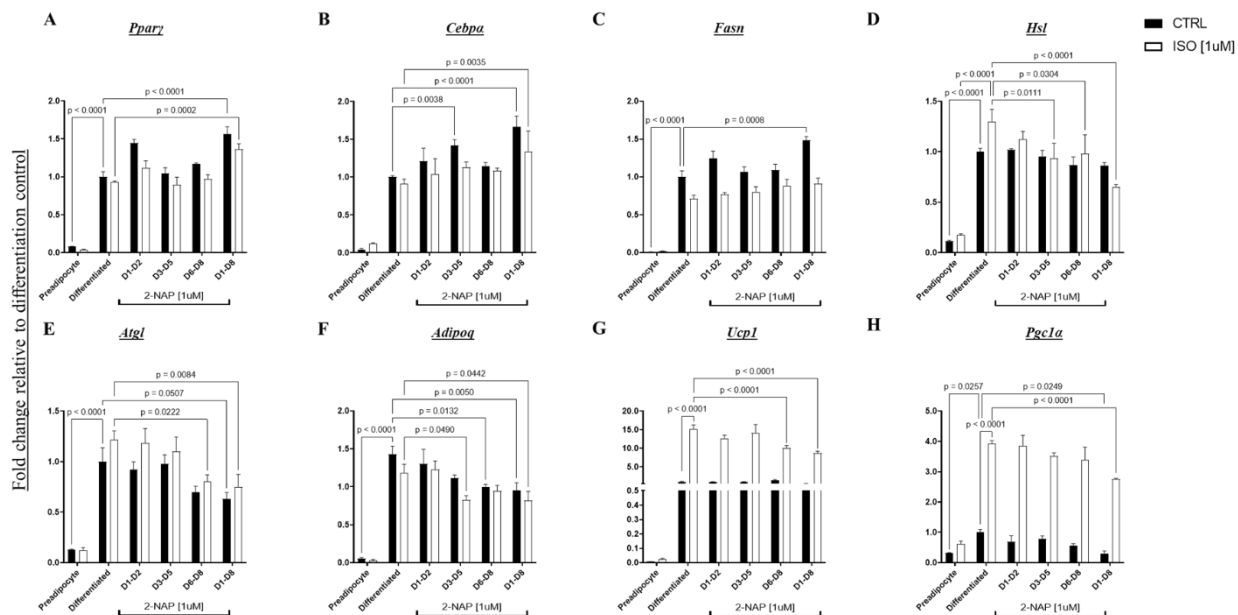
**Figure 2.5 Chronic 2-NAP exposure increases expression of inflammatory genes in 3T3-L1 cells.**

Relative mRNA expression of inflammatory genes (*Tnfa*, *Il-1 $\beta$* , and *Il-6*) in 3T3-L1 cells chronically treated with 1  $\mu$ M 2-NAP as indicated. LPS (1ng/ml) was used as a positive control. Upon experimental termination, mRNA was extracted, and relative gene expression was then

determined by RT-PCR (n = 4). The values shown represent the mean  $\pm$  SEM. A *p*-value of *p* < 0.05 indicates statistical significance between two groups.

#### ***2.4.7 Brown adipocytes exposed to 2-NAP during differentiation displayed a decrease in thermogenesis and an increase in adipogenic gene expression***

To determine the role of LMW-PAH exposure in the regulation of thermogenesis and brown-fat-specific gene expression, BAT1 cells were chronically exposed to 2-NAP at different points of differentiation from days 1-2, 3-5, 6-8, and 1-8. On day 8 of differentiation, BAT1 cells were treated with 1 $\mu$ M of the  $\beta$ -adrenergic agonist isoproterenol (ISO) for 4 hours before cells were harvested for gene expression analysis. As expected, differentiated BAT1 brown adipocytes exhibited significantly increased expression of genes in adipocyte differentiation and thermogenesis compared to that of preadipocytes, including markers of adipocyte differentiation *Ppar $\gamma$*  and *Cebpa*, lipogenesis marker *Fasn*, lipolysis markers *Hsl* and *Atgl*, adipokine *Adipoq*, and thermogenesis markers uncoupling protein 1 (*Ucp1*) and PPARG coactivator 1 alpha (*Pgcl $\alpha$* ) (Fig. 2.6). ISO-treated BAT1 cells showed increased expression of thermogenic and lipolytic markers *Ucp1*, *Pgcl $\alpha$* , and *Hsl* (Fig. 2.6). Similar to 3T3-L1 cells, BAT1 cells treated with 2-NAP across the entire cellular differentiation process (days 1– 8) showed increased expression of adipogenesis genes, including the adipocyte differentiation markers *Ppar $\gamma$*  and *Cebpa* and the lipogenesis marker *Fasn*. 2-NAP treatment across all measured differentiation points resulted in significant downregulation of *Adipoq* (Fig. 2.6). BAT1 brown adipocytes treated with 2-NAP during days 1-8 of differentiation resulted in a downregulation in basal expression of *Pgcl $\alpha$*  and a tendency of downregulation in basal expression of *Atgl*. Furthermore, chronic 2-NAP treatment during BAT1 cell differentiation significantly down-regulated ISO-stimulated *Ucp1*, *Pgcl $\alpha$* , *Hsl*, and *Atgl* expression (Fig. 2.6).



**Figure 2.6 Effect of 2-NAP on thermogenic and adipogenic genes in BAT1 cells.**

Relative mRNA expression of adipogenic transcription factors *Pparγ* (A) and *Cebpa* (B); the adipocyte specific marker *Fasn* (C); markers of lipogenesis and lipolysis *Hsl* (D), and *Atgl* (E); the adipokine *Adipoq* (F); and thermogenic genes *Ucp1* (G) and *Pgc1α* (H) was measured in BAT1 cells chronically treated with 1  $\mu$ M 2-NAP. On day 8, half of the cells were further treated with 1  $\mu$ M isoproterenol (Iso) for 4 h. Upon experimental termination, mRNA was extracted, and relative gene expression was then determined by RT-PCR (n = 4). The values shown represent the mean  $\pm$  SEM. A *p*-value of  $p < 0.05$  indicates statistical significance between two groups.

## 2.5 Discussion

We are exposed daily to polycyclic aromatic hydrocarbons (PAH). Researchers have previously discovered associations between the presence of PAH metabolites in our body and the incidence of obesity and obesity-related comorbidities (Diggs et al., 2011; Marris et al., 2020; Scinicariello & Buser, 2014). Most studies focus on HMW-PAH; however, little is known about the effects of individual LMW-PAHs like naphthalene, which is more pervasive and easier metabolized into bioactive compounds (1-naphthol and 2-NAP). *In vivo* and *in vitro* studies have revealed the obesogenic potential LMW-PAHs like 2-NAP (Agency for Toxic Substances and Disease Registry

(ATSDR), 2005; Kilanowicz et al., 1999). On the basis of this previous research, we aimed to identify the cellular pathways LMW-PAHs may influence, contributing to metabolic dysfunction associated with obesity. This work investigated how 2-NAP exposure impacts adipocyte and macrophage physiologies using RAW 264.7, 3T3-L1, and BAT1 murine cell lines to model key regulators of adipose tissue health. These *in vitro* models represent macrophages, white adipocytes, and brown adipocytes, respectively. These models allowed us to analyze the effect of 2-NAP on the overall inflammatory status and at distinct stages of adipocyte differentiation. Using these models, we investigated 1) the role of 2-NAP on adipocyte function and lipid metabolism; and 2) the impact of 2-NAP exposure on adipose inflammation.

Adipogenesis, the process by which new adipocytes are generated, is defined by sequences of cellular recruitment, expansion, differentiation, and maturation of committed preadipocytes (Berry et al., 2013). Distinct transcriptional events mark these developmental stages. Key transcription factors, *Ppar $\gamma$*  and *Cebpa*, are up-regulated during early differentiation. This research reveals that *Ppar $\gamma$*  and *Cebpa* mRNA are significantly increased in white adipose and brown adipose tissues chronically exposed to 2-NAP (Fig 2.3. and Fig 2.6.) The stimulation of these transcription factors activates a battery of other target genes during late differentiation, including aP2, a marker specific to mature adipocytes, lipogenic marker *Fasn*, lipolysis markers *Hsl* and *Atgl*, and the adipokine *Adipoq*. Interestingly, we found that 2-NAP treatment during 3T3-L1 white adipocyte differentiation and BAT1 brown adipocyte differentiation significantly stimulated the expression of *Ppar $\gamma$*  and *Cebpa*. In addition, 2-NAP treatment significantly increased the expression of *Fasn*, an important enzyme involved in lipogenesis, and coordinately downregulated the expression of *Hsl* and *Atgl*, enzymes involved in lipolysis. This coordinated regulation in adipogenic, lipogenic,



and lipolytic gene expression could contribute to the increased lipid accumulation observed in 2-NAP treated adipocytes (Fig. 2.4), which supports our hypothesis of 2-NAP as a potential obesogen. Interestingly, we found that continuous 2-NAP presence rather than transient treatment during the differentiation process may be required for 2-NAP's obesogenic function, as transient treatment of 2-NAP during the early and mid-stage of differentiation did not enhance lipid accumulation in either 3T3-L1 white adipocytes or BAT1 brown adipocytes.

The observed decrease in *Adipoq* mRNA expression aligns with previous work showing decreased *Adipoq* mRNA expression in ob/ob mice compared to lean ob/+ mice (Hu et al., 1996). Furthermore, serum adiponectin levels in wild-type mice on a high-fat diet or db/db mice are decreased compared with control animals (Yamauchi et al., 2001). Similar trends of *Adipoq* dysregulation were discovered upon examination of the plasma from obese humans (Arita et al., 1999). Adiponectin has been shown to contribute to a beneficial metabolic phenotype through various functions, including suppressing hepatic glucose production, stimulating fatty acid oxidation in muscle, and inhibiting inflammation in various tissues (Stern et al., 2016; Z. V Wang & Scherer, 2016). Thus, inhibiting adiponectin (*Adipoq*) expression in both 3T3-L1 white adipocytes and BAT1 brown adipocytes using 2-NAP may contribute to the impact of 2-NAP on metabolic dysregulation.

While the function of white adipocytes is to store excess energy as fat, that of brown adipocytes is to dissipate energy as heat through adaptive thermogenesis due to its unique expression of UCP1 in the mitochondria inner membrane (Kajimura et al., 2010; W. Wang & Seale, 2016). UCP1 acts to uncouple oxidative phosphorylation from ATP synthesis, dissipating energy as heat and

profoundly increasing overall energy expenditure (Cannon & Nedergaard, 1985, 2011). Brown adipocyte thermogenesis is regulated by catecholamine signaling. Catecholamines released by sympathetic nerve terminals in response to cold stimulate lipolysis and activate BAT/beige thermogenesis via  $\beta$ -adrenergic receptors (Cannon & Nedergaard, 2004; Cinti, 2005). Our findings showed that 2-NAP significantly suppressed the  $\beta$ -adrenergic receptor agonist isoproterenol-stimulated *Ucp1* expression in BAT1 brown adipocyte cells, which may contribute to its obesogenic potential.

Obesity is associated with a chronic low-grade inflammatory state, and adipose tissue inflammation is causatively linked to obesity-associated metabolic disorders, including insulin resistance and type 2 diabetes (Hotamisligil, 2006; Olefsky & Glass, 2010). An essential feature of obesity-induced inflammation is the infiltration of macrophages into adipose tissue, which, together with inflamed adipocytes, contributes to obesity-associated chronic inflammation (Weisberg et al., 2003; Xu et al., 2003). We found that chronic 2-NAP treatment in macrophages and adipocytes significantly increased the expression of proinflammatory cytokines. Thus, chronic exposure to the obesogen 2-NAP may further precipitate metabolic disorders by enhancing macrophage and adipocyte inflammation.

Overall, our findings show that 2-NAP significantly enhances adipocyte lipid accumulation by up-regulating adipogenic (*Ppar $\gamma$* , *Cebpa*) and lipogenic (*Fasn*) biomarkers while downregulating markers of lipolysis (*Hsl*, *Atgl*) in a differentiation stage-dependent fashion. In addition, we found that 2-NAP downregulates adiponectin expression in white and brown adipocytes and inhibits ISO-stimulated thermogenic expression in brown adipocytes. Moreover, 2-NAP also significantly

stimulates proinflammatory cytokine expression in white adipocytes and macrophages. Data collected from this study will help characterize the role of PAHs in metabolism, obesity, and inflammation. Further characterization of this pathway may have significant implications for developing preventative and therapeutic strategies for many metabolic disorders.

### 3 SPECIFIC AIM 2: *IN VIVO* EFFECTS OF CHRONIC 2-NAPHTHOL EXPOSURE IN THE INVERTEBRATE *DAPHNIA MAGNA*.

#### 3.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants. An examination of their chemical composition and evidence from previous research suggests that they may act as endocrine disruptors. The physiological implications of PAH exposure have been linked to declines in fecundity, maternal health, and offspring development (Incardona et al., 2004; Jeong et al., 2016; Rundle et al., 2012). Evidence linking maternal exposure to the PAH, 2-naphthol (2-NAP), and the occurrence of childhood obesity elicits the exploration of 2-NAPs potential to propagate negative effects across multiple generations. Recent studies have demonstrated *Daphnia magna* to be a reliable model for studying lipid dynamics *in vivo* (R. Jordão et al., 2015, 2016; Poynton et al., 2007; Shaw et al., 2008; Zou & Fingerman, 1997). This study aimed to assess the effects of chronic 2-NAP exposure on *D. magna* across multiple generations. Specifically, this study sought to 1) investigate the influence of 2-NAP on *D. magna* life-history and fitness traits; 2) monitor the generational effects of 2-NAP in offspring whose mothers were exposed during the egg provisioning stage, and 3) evaluate the effects of 2-NAP on lipid accumulation across all experimental groups. The production of *in vivo* data could provide valuable insight into identifying the molecular targets of other individual LMW-PAHs associated with obesity and related metabolic disorders.

2-NAP is a polycyclic aromatic hydrocarbon (PAH) that was found to be related to higher body mass index in children (Scinicariello and Buser, 2014). It also stimulates early differentiation of 3T3-L1 preadipocytes *in vitro*. However, the *in vivo* effects of 2-NAP have not been fully explored.

Using *D. magna* as *in vivo* model, we wish to examine the effects of 2-NAP on lipid homeostasis and life-history traits across the parental (F0), first filial (F1), and possibly future generations. There are many advantages to using daphnia as a biological model, such as their ability to produce asexual clones and short reproductive cycles. We plan to expose the daphnia to varying concentrations of 2-NAP. This will allow us to analyze fitness, metabolic, and generational consequences across treatments and control groups. Like most living species, lipids are the daphnia's primary source of energy for metabolic and reproductive maintenance (Martin-Creuzburg & Elert, 2004). The disturbance of lipid production and trafficking will likely influence high-energy processes like reproduction and molting (Goulden & Place, 1993; R. G. Jordão et al., 2016; Wacker & Martin-Creuzburg, 2007). We hypothesize that 2-NAP will increase lipid accumulation in daphnia. This exposure will likely result in lipid retention in the mother during offspring development and release. Evaluating the effects of 2-NAP on lipid homeostasis in an *in vivo* model is the first step to identifying underlying mechanisms that are disturbed due to 2-NAP toxicity. This study will also allow us to examine the generational effects of 2-NAP, even after direct exposure is removed.

## **3.2 Materials and methods**

### ***3.2.1 Chemicals***

2-Naphthol (2-NAP; CAS No. 135-19-3) was purchased from ACROS Organics. Nile Red (CAS No. 7385-67-3) was purchased from Sigma-Aldrich. Culture medium was prepared according to standards established by the U.S. Environmental Protection Agency Office of Water (EPA, 2002). Chemicals used for synthetic freshwater (USEPA 2002) were purchased from Sigma-Aldrich as

well; Sodium Bicarbonate (CAS No. 144-55-8), Potassium Chloride (CAS No. 7447-40-7), Magnesium Sulfate (CAS No. 7487-88-9), Calcium Sulfate (CAS No. 10101-41-4).

### **3.2.2 Experimental animals and design**

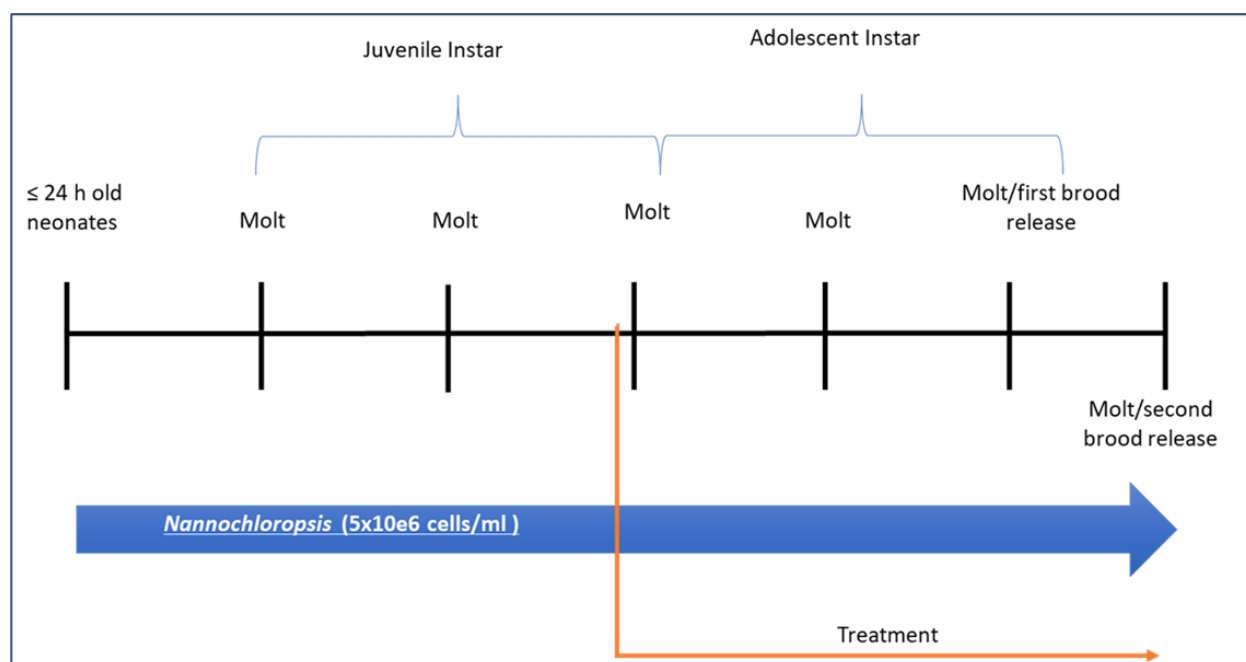
Understanding the daphnia life cycle is the most critical aspect of using this organism as a model to assess metabolic health. At 4-8 hrs old, daphnia are considered to be neonates. The third juvenile instar begins 4-8 hours before molting for the third time. The adolescent instar begins around 3 days. *D. magna* usually reach adulthood at 5 days and can live up to 2 months under adequate feeding conditions. Females can reproduce every 3 to 4 days until death. The resting eggs (ephippia) can be kept for several years, providing maximum flexibility. Females keep their eggs and recently hatched young in their brood chambers for several days, providing nutrients during development. Once juveniles are released, there is no additional parental care (Ebert, 2005). All experiments were performed using *D. magna* reared in the lab indefinitely as pure parthenogenetic cultures (Carolina Biological Supply Company, Burlington, NC). All experiments were initiated using synchronized neonates less than 24 hours old. Individual cultures, groups of 15 animals, were maintained in 250 mL of hard synthetic freshwater under high food conditions (*Nannochloropsis spp.*  $5 \times 10^5$  cells/ mL). Feeding standards were adapted from previous food studies (Barata & Baird, 1998; Wacker & Martin-Creuzburg, 2007). We also have to consider the possibility of phenotypic plasticity between genetically identical clones (Barata & Baird, 1998).(Barata & Baird, 1998).(Barata & Baird, 1998).(Barata & Baird, 1998). Meaning, inconsistencies in output measures, such as offspring size and survival rates, cannot always be accounted for by metabolic/chemical disturbances. We address this challenge by only using clones reared from reproductively experienced adults. Adult Daphnia that have released more than three

clutches tend to produce more homogenous offspring throughout their adult life (Dudycha & Tessier, 1999).

Because the previous work was done using mouse cells, it is crucial to consider parameters that fit our current model (*D. magna*) when selecting treatment concentrations. (Offem & Ayotunde, 2008) reported 24h, 48h, and 964h EC50 concentrations for lead exposure as 7.72  $\mu\text{M}$ , 5.78  $\mu\text{M}$ , and 5.07  $\mu\text{M}$  mg/l for *D. magna*, respectively. Other labs have reported similar EC50 values for both acute and chronic lead exposure (Altinda et al., 2008; Cooper et al., 2009). Millemann et al., (1984) reported 2-NAP as toxic at 25  $\mu\text{M}$ . All values mentioned correlate with concentrations that either kill or immobilize 50% of the test population in the allocated time. With this knowledge, it is important to choose parameters that align with our previous work and falls below the toxicity threshold of the *D. magna* model. 2-NAP concentrations of 0.1  $\mu\text{M}$ , 1  $\mu\text{M}$ , 10  $\mu\text{M}$ , and 20  $\mu\text{M}$  were selected for the first experimental run (not shown). These concentrations allow us to test within the range of previous work and below levels toxic to daphnia. Animals exposed to the 20  $\mu\text{M}$  concentration died at 6 days old. We eliminated the 20  $\mu\text{M}$  concentration due to the acute toxicity displayed in our previous system. Additionally, the 20  $\mu\text{M}$  treatment group did not develop eggs within the brood pouch (not shown). Millemann et al., (1984), described 2-NAP being acutely toxic in *D. magna* at 25  $\mu\text{M}$  after a 48-hour exposure period, corroborating the toxic effects we discovered at 20  $\mu\text{M}$ .

Chemical treatment began before the third molt (~72 h), preceding egg development. Animals were exposed to five 5-fold concentrations of 2-NAP, ranging from 0.016  $\mu\text{M}$  to 10  $\mu\text{M}$ . Treatment groups were compared to a negative control (without chemical treatment) and solvent control:

Negative control – (US Environmental Protection Agency, 2002) synthetic hard water + *Nannochloropsis* at  $5 \times 10^5$  cells/ml; Solvent control - 0.0125% ethanol (solvent used for the highest 2-NAP exposure concentration). Throughout the treatment period, we collected daily fitness and life-history data. Upon termination (day 14), animals were sacrificed for lipid extraction and quantification. A schematic of the treatment protocol is shown in Fig. 3.1. The experiment was terminated after the control group released their second clutch of neonates.



**Figure 3.1 Treatment Protocol.**

### 3.2.3 Individual fitness and life-history traits

Fitness traits output measures include clutch size (average number of neonates per subject) and survival rates (percent of viable subjects). Life-history traits were evaluated by recording molting events (average number of molts per subject), the first appearance of eggs (days), and the age at the first and second brood release (days). All parameters were monitored starting from the neonate stage ( $\leq 24$  h) and following through the juvenile ( $\sim 3$  days) and adult ( $\sim 3 - 14$  days) stages. Daphnia



were followed throughout two consecutive clutch releases, as was the second F1 generation (F1-2) produced by the mothers exposed to 2-NAP. Immediately upon hatching, 15 randomly selected F1-2 neonates from each group were transferred to 2-NAP-free media (synthetic hard water + *Nannochloropsis* at  $5 \times 10^5$  cells/ml only).

Methods used to collect clutch size and the first appearance of eggs limit thorough analysis of these parameters. Because the *Daphnia* are cultured in groups of 15, it is nearly impossible to collect this data for each individual animal within one treatment group without removing the animals from the medium. When culturing *daphnia*, excessive and rough handling can stress the animals and result in death. As a result, *daphnia* are only handled when being transferred to new media — making it only possible to report descriptive statistics on molting events, age at first reproduction, age at second reproduction, and lipid quantification data. Individual observations of egg appearance could not be recorded, and egg appearance was characterized by the day on which more than half the sample population had eggs in the brood pouch.

#### ***3.2.4 Lipid quantification***

Nile Red is a dark purplish-red powder (Sigma N-3013). The stock solution must be prepared in acetone (500  $\mu\text{g/ml}$ ) and kept in a tightly sealed, lightproof container at 4 degrees. Reconstitute the 10 mg of Nile Red in 20.0 mL of acetone to achieve a final concentration of 500  $\mu\text{g/ml}$  (157 mM). Aliquot stock solution in volumes of 0.5 mL and store in the  $-20\text{ }^\circ\text{C}$  freezer. From one stock solution, make an intermediate solution of 157  $\mu\text{M}$  by adding 100  $\mu\text{L}$  of the 1.57 mM stock solution to USEPA 2002 to make a final volume of precisely 1 mL. The working solution should be prepared by diluting the stock solution to 1.5  $\mu\text{M}$  in ASTM. Animals were pooled in groups of ten

daphnia per treatment group for Nile Red staining, extraction, and analysis. Live individuals were exposed to the Nile Red working solution in the dark for 1 hr at 20°C. After incubation, animals were placed in 100 mL of USEPA 2002 for 1 min to allow clearance of Nile Red residuals. To extract the Nile Red stain from lipids, 10 adult females from each experimental group were placed in 1.5-mL centrifuge tubes and sonicated in 600  $\mu$ L of isopropanol. The homogenized extract was incubated in isopropanol for 15 mins, then centrifuged at  $10,000 \times g$ . 150  $\mu$ L of extract supernatant delivered, in triplicates, into a 96-well plate. We then measure Nile Red fluorescence at an excitation/emission wavelength of 548/600 nm using the EnSpire microplate reader.

### ***3.2.5 Statistical analysis***

The statistical significance of 2-NAP exposure on Nile Red fluorescence and fitness responses was analyzed by one-way analysis of variance (ANOVA). If statistical significance was established using one-way ANOVA, post hoc Tukey's or Dunnett's tests were performed to further compare individual relationships between chemical treatment groups and the respective controls. Tests were selected based on variance and sample size equality. Significance levels are denoted using asterisks:  $p < 0.05 = *$ ;  $p < 0.01 = **$ ;  $p < 0.001 = ***$   $p < 0.0001 = ****$ .

## **3.3 Results**

### ***3.3.1 Daphnia exposed to 2-NAP show a decrease in overall fitness.***

Chronic 2-NAP exposure significantly increased the average age of first and second brood release compared to the control. Both brood release and the first appearance of eggs were delayed by almost 1 day (Table 3.1). The number of offspring produced by the F0 generation is also lower in all treatment groups. All fitness and life history data collected for the first experimental run is

presented in Table 3.1. The F0 generation showed statistically significant increases in ages at first and second brood releases. A modest reduction in clutch size was the only noticeable effect of 2-NAP exposure on the reproductive activity of the F1-2 generation. All other reproductive functions seemed to be unaffected. However, the chance of Daphnia from the F1-2 generation surviving to adulthood (15 days) is significantly reduced compared to controls and the parental generation.

During the second experimental run, animals were exposed to five 5-fold concentrations of 2-NAP, ranging from 0.016 to 10  $\mu$ M. We eliminated the 20  $\mu$ M concentration due to its acute toxicity displayed in our system. It also appears that 2-NAP is slightly toxic at the 10  $\mu$ M concentration, as shown by the reduction in survival rates across both generations (Table 3.1).

The F1-2 generation was also followed for 15 days. Fitness and life history traits for both generations of the second experimental run are shown in Table 3.1. There were significant ( $p < 0.05$ ) differences among several traits across 2-NAP treatments compared to controls. The F0 generation showed statistically significant increases in ages at the first and second brood releases. A modest reduction in clutch size was the only noticeable effect of 2-NAP exposure on reproductive activity of the F1-2 generation. All other reproductive functions seemed to be unaffected. However, the chance of daphnia from the F1-2 generation surviving to adulthood (15 days) is greatly reduced when compared to controls and the parental generation.

Traits	Negative Control	Solvent Control	0.016 $\mu$ M 2-NAP	0.08 $\mu$ M 2-NAP	0.4 $\mu$ M 2-NAP	2 $\mu$ M 2-NAP	10 $\mu$ M 2-NAP
<b>Parental generation (F0)</b>							
Adult survival to 15 days (%)	100.00	100.00	100.00	100.00	100.00	100.00	73.33
First appearance of eggs (days)	4	4	4	4	5	5	5
Age at first brood release (days)	7.250 $\pm$ 0.447	7.313 $\pm$ 0.479	7.214 $\pm$ 0.426	7.333 $\pm$ 0.617	8.067 $\pm$ 0.258***	8.071 $\pm$ 0.267***	8.133 $\pm$ 0.352***
Age at second brood release (days)	11.13 $\pm$ 0.342	11.19 $\pm$ 0.403	11.64 $\pm$ 0.497**	11.87 $\pm$ 0.352***	11.93 $\pm$ 0.457***	11.86 $\pm$ 0.363***	12.13 $\pm$ 0.516***
<b>Clutch size (n)</b>							
F1-2	132	121	86	88	87	72	56
<b>First generation (F1-2)</b>							
Adult survival to 15 days (%)	100.00	100.00	100.00	87.50	86.67	66.67	60.00
First appearance of eggs (days)	4	4	4	4	4	5	5
Age at first brood release (days)	8.143 $\pm$ 363	8.067 $\pm$ 0.258	8.133 $\pm$ 0.352	8.067 $\pm$ 0.258	8.133 $\pm$ 0.352	8.267 $\pm$ 0.458	8.400 $\pm$ 0.507
Age at second brood release (days)	11.14 $\pm$ 0.363	11.13 $\pm$ 0.352	11.07 $\pm$ 0.258	11.13 $\pm$ 0.352	11.20 $\pm$ 0.414	11.27 $\pm$ 0.458	11.27 $\pm$ 0.458
<b>Clutch size (n)</b>							
F2-2	139	134	119	122	127	126	118

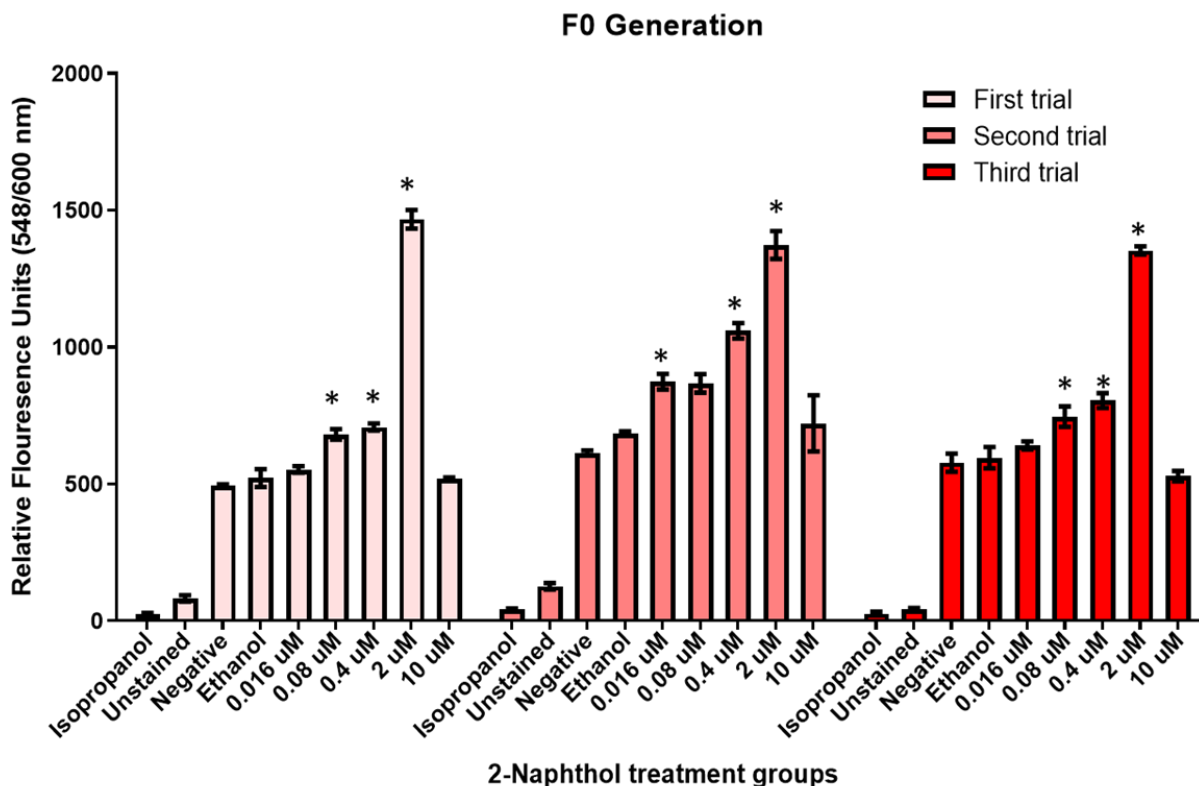
**Table 3.1. Summary of fitness traits.**

Values of fitness traits are listed as means  $\pm$  SE; n=16 for negative control; n= 16 for solvent control; n=14 0.016 $\mu$ M 2-NAP treatment group; n=14 for 0.08 $\mu$ M 2-NAP treatment group; n=15 for 0.4 $\mu$ M treatment group; n=14 for 2  $\mu$ M treatment group; n=15 for 10  $\mu$ M treatment group. 0.0125% ethanol was used as the solvent control. 2-naphthol (2-NAP). Significance levels are denoted using asterisks:  $p < 0.05 = *$ ;  $p < 0.01 = **$ ;  $p < 0.001 = ***$   $p < 0.0001 = ****$ .

### 3.3.2 *Daphnia* show a dose-dependent increase lipid accumulation in response to 2-NAP.

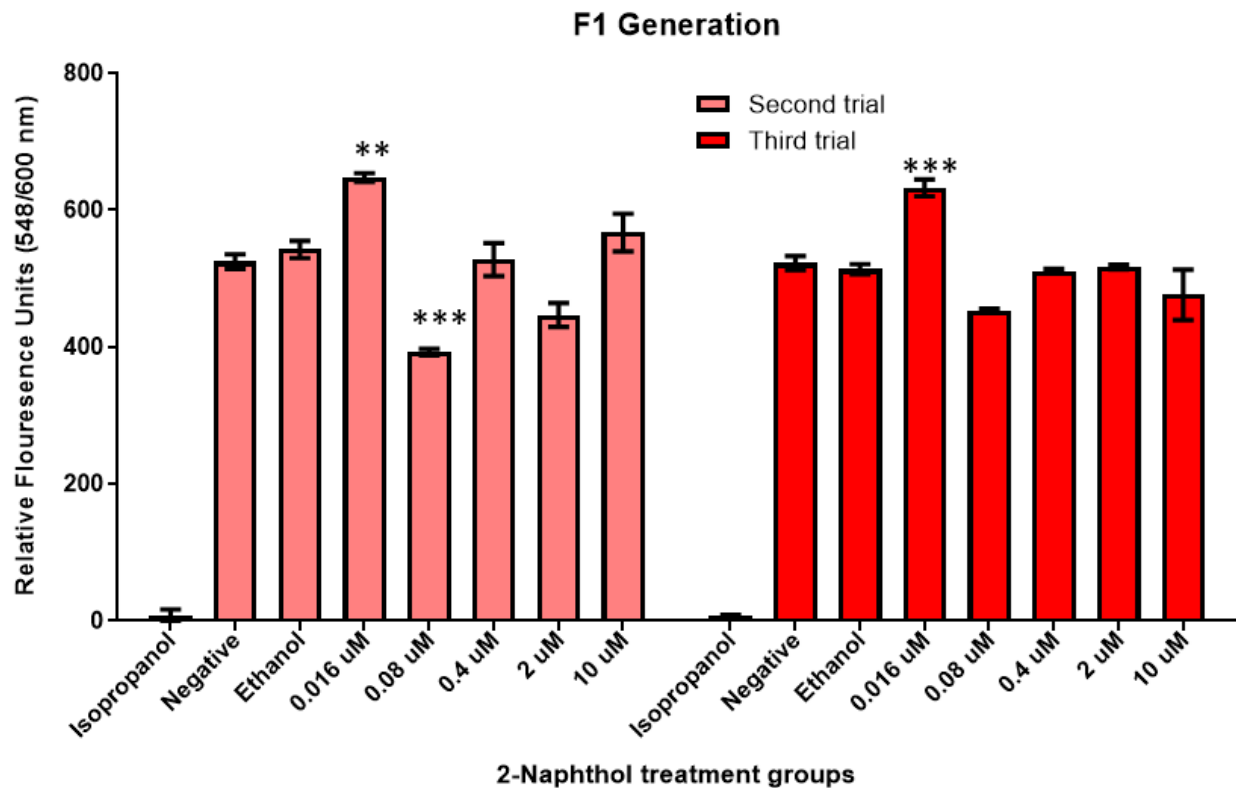
The next set of experiments employed the use of the Nile Red staining to measure the effects of 2-NAP exposure on lipid production in *D. magna*. Triacylglycerols are the major group of storage lipids in daphnia (Goulden & Place, 1993). Findings from (R. Jordão et al., 2015) showed that quantification of lipid droplets using Nile Red fluorescence is highly correlated to the total levels of triacylglycerols present. Figure 3.2 shows a dose-dependent increase in overall lipid accumulation following direct 2-NAP exposure with significant increases between the 0.08 – 2.00

µM range. This is represented by an increase in Nile Red fluorescence as a response to increasing 2-NAP concentrations. Although the mothers exposed to 2-NAP produced offspring with reduced fitness, the effects of 2-NAP on lipid accumulation in the F1 generation are not displayed in a dose-dependent manner (Fig. 3.3).



**Figure 3.2. Nile red staining of lipid droplets in *D. magna*.**

Each bar and line represents three replicates' average RFU  $\pm$  standard deviation. The statistical significance of 2-NAP exposure on Nile Red fluorescence and fitness responses was analyzed by one-way analysis of variance (ANOVA). If statistical significance was established using one-way ANOVA, a post hoc Tukey's tests was performed to further compare individual relationships between chemical treatment groups and the respective controls. The test was selected based on sample variance and sample size equality. Significance levels are denoted using asterisks:  $p < 0.05$  = \*.



**Figure 3.3. The metabolic effects of 2-naphthol are not conserved across generations.**

Each bar and line represents three replicates' average RFU  $\pm$  standard deviation. The statistical significance of 2-NAP exposure on Nile Red fluorescence and fitness responses was analyzed by one-way analysis of variance (ANOVA). If statistical significance was established using one-way ANOVA, a post hoc Tukey's tests was performed to further compare individual relationships between chemical treatment groups and the respective controls. The test was selected based on sample variance and sample size equality. Significance levels are denoted using asterisks:  $p < 0.01 = **$ ;  $p < 0.001 = ***$ .

Molting frequency for the two parental F0 generations is plotted as the total number of molts over time (days). The first experimental run shows an apparent increase in molting frequency compared to controls (not shown). There was a similar increase in molting frequency for the second experimental run, however only for the 0.4 uM, 2 uM, and 10 uM concentrations (not shown). Daphnia exposed 2-NAP concentrations greater than or equal to 0.1 uM appeared to molt more often when compared to controls (not shown).

### 3.4 Discussion

PAHs are environmental pollutants that can be found virtually everywhere throughout the environment. Epidemiological evidence linking maternal exposure to chemicals, such as 2-NAP, with childhood obesity, raises concerns about its long-term (generational) effects. This study examined the adverse effects of direct and indirect 2-NAP exposure in *Daphnia magna*. We choose the *D. magna* model to explore fitness, life-history, and lipid dynamics across the parental (F0) and first filial (F1) generations. Data revealed that direct 2-NAP exposure could alter reproductive-related events. Animals directly exposed to 2-NAP molt more frequently and show delayed reproductive development. They even produce fewer offspring during brood development compared to controls. With the exception of offspring production, reproductive functions of the F1 generation are not significantly affected by prenatal 2-NAP exposure. However, prenatal exposure to 2-NAP drastically reduced survival rates in the F1 generation, even after chemical removal. There appears to be a dose-dependent increase in overall lipid quantity following direct 2-NAP exposure. However, this relationship disappears in the succeeding generation when the chemical is removed. Offspring of the F0 daphnia exposed to the lowest concentration of 2-NAP show an increase in lipid levels. While offspring of F0 daphnia exposed to the remaining concentrations show either lower or no change in lipid levels.

Being that 2-NAP is a known hormone agonist, one possible explanation for its effects in *D. magna* could be that 2-NAP is interfering at some level in the ecdysteroid pathway. Ecdysteroids are hormones that are essential for the growth and reproduction of insects and other arthropods. Known agonists of ecdysteroid hormones can regulate growth, reproductive-related mechanisms and lipid distribution (Martin-Creuzburg & Elert, 2004; Wacker & Martin-Creuzburg, 2007). In

daphnia, RXR is activated by the binding of ecdysteroids to the receptor. When activated, RXR forms a complex with other transcription factors, such as the ecdysone receptor (EcR), and bind to specific DNA sequences known as ecdysone response elements (EcREs). This binding leads to the transcription of target genes, which are involved in various processes such as development, molting, and reproduction. Jordao et al. (2015), demonstrated that daphnia exposed to the obesogen, TBT, exhibited increased expression of RXR mRNA (R. Jordão et al., 2015). RXR expression was directly correlated to molting events and lipid production. The F0 generation showed an earlier release of offspring and a reduction in clutch size. This suggests that 2-NAP could be influencing RXR activity in the daphnia. The accumulation of lipids we observed in the F0 generation is most likely due to the retention of reservoirs usually allocated to offspring development. This would explain why 2-NAP exposure reduced offspring (F1) lifespan and survival. Molting frequency is another event regulated by lipid metabolism and transcription factors such as RXR. Further characterization of this pathway could have significant implications in developing preventative and therapeutic strategies for many metabolic disorders.

Furthermore, our data suggest the possibility of 2-NAP exposure having generational consequences. Generational effects induced by environmental cues often leave epigenetic marks that can be passed down. The consequence of epigenetic modulations can sometimes skip generations or may not manifest until late developmental stages. Future studies could include increasing the number of examined generations and following the animals throughout their life cycle. Future research will be required to establish the precise molecular pathways behind 2-NAP's effects on fecundity, offspring survival, and lipid homeostasis.



Overall, our findings suggest that 2-NAP reduces individual fitness and offspring survival in both the parental (F0) and first filial generations (F1). 2-NAP can also alter reproductive functions and molting patterns, two major events that are regulated by hormonal pathways. The findings of this study add to the evidence that 2-NAP is an endocrine disruptor that contributes to the development of obesity and associated metabolic problems.

#### 4 SUMMARY AND CONCLUSION

Every day we are exposed to polycyclic aromatic hydrocarbons (PAH). Over the years, researchers have found significant associations between the presence of PAH metabolites in our bodies and the incidence of obesity and its comorbidities. Both low-molecular-weight PAHs (LMW PAHs) and high-molecular-weight PAHs (HMW PAHs) have been shown to have potential health effects. However, there is evidence that LMW PAHs may be more biologically active and have a greater potential to cause adverse health outcomes, including obesity, than HMW PAHs. HMW PAHs, on the other hand, are made up of larger molecules and are less easily absorbed by the body. They are generally less biologically active than LMW PAHs. While HMW PAHs can still have negative health effects, and exposure to high levels of these chemicals has been linked to an increased risk of cancer, there is less evidence showing their individual contribution to obesity. A large host of research focuses on the biochemical effects of high-molecular-weight (HMW) PAHs, such as benzo(a)pyrene. However, when the urinary PAH content of obese children and their non-obese counterparts were compared, low-molecular-weight (LMW) PAHs showed stronger correlations with childhood obesity, higher BMI, and larger waist circumferences. Naphthalene, structurally the simplest PAH, not only showed the strongest association with childhood obesity but also the most concentrated urinary compound detected in all samples (Scinicariello & Buser, 2014). LMW PAHs are smaller molecules and are more readily metabolized by the body than HMW PAHs. In addition, LMW PAHs have been shown to alter the metabolism of fat cells and increase fat accumulation in the body, which can contribute to obesity. Understanding the relative contribution of LMW PAHs and HMW PAHs to obesity is important because it can help to identify potential strategies for preventing or reducing exposure to these chemicals. By focusing on LMW PAHs, it may be possible to more effectively target interventions to reduce the risk of obesity and related

health problems. To examine the effects of PAH on the development of obesity and related metabolic disorders, we analyzed data generated using the *D. magna* (invertebrate) model alongside adipocyte and macrophage cellular (vertebrate) models. We used these findings to help disentangle complex interactions between 2-NAP exposure and various metabolic mediators of lipid homeostasis.

The first study examined the influence of 2-NAP on adipocyte function, lipid metabolism, and metabolic inflammation. Liver and adipose tissues are among the first to display abnormalities during the development and progression of metabolic disorders. Macrophage infiltration of WAT is a hallmark characteristic of the chronic low-grade inflammation activated during the obese state (Ferrante, 2013). This chronic inflammation is then propagated by the hyper-stimulation of resident adipose tissue macrophages (ATMs) by endogenous and/or exogenous stimuli. ATMs are the primary producers of TNF- $\alpha$  and of IL-6 in WAT (Ahima & Flier, 2000). The production of these cytokines is positively associated with insulin resistance and obesity. Adding adipogenic chemicals, such as PAHs, to this cycle, exaggerates these effects even more. To determine the effect of PAH exposure on the overall state of adipocyte and macrophage inflammation, we used quantitative RT-PCR to measure the expression of mRNA transcripts of metabolic and inflammatory-associated genes. We found that prolonged exposure of RAW 264.7 macrophages as well as differentiated 3T3-L1 adipocytes to 2-NAP significantly increased the expression of *Tnfa*, *Il-1 $\beta$* , and *Il-6* mRNA transcript levels and significantly enhanced LPS-induced inflammatory gene expression. Additionally, we found that 2-NAP treatment significantly upregulated the expressions of *Ppar $\gamma$* , *Cebpa*, *Fasn*, while downregulating *Hsl*, and *Atgl* in a differentiation stage-dependent fashion. We also found that 2-NAP significantly downregulated

the  $\beta$ -adrenergic receptor agonist isoproterenol-stimulated *Ucp1* and other thermogenic gene expression in BAT1 cells. The findings of this study suggest that 2-NAP may contribute to metabolic dysfunction associated with obesity through its effects on adipogenesis, inflammation, and lipid metabolism.

We also investigated the effects of 2-NAP on *D. magna in vivo* across multiple generations, focusing on 1) how it influences life-history and fitness traits. 2) generational effects of 2-NAP in offspring whose mothers were exposed during the egg provisioning stage. 3) and the effects of 2-NAP on lipid accumulation across all experimental groups. Our findings suggest that exposure to 2-NAP significantly disturbs overall *D. magna* fitness. *Daphnia* exposed to higher concentrations release their broods later than control animals. Offspring fitness was also reduced, even upon removal of direct 2-NAP exposure. There is evidence of a dose-dependent increase in overall lipid accumulation following direct 2-NAP exposure. This data is consistent with work showing 2-NAP exposure accelerated pre-adipocyte differentiation and lipid accumulation of mouse 3T3-L1 cells. The results of this study suggest that 2-NAP exposure can have negative effects on *D. magna*, including altered reproductive-related events, decreased fecundity, and increased lipid accumulation.

Overall, our findings suggest that LMW-PAHs may contribute to metabolic dysfunction associated with obesity. *In vivo* and *in vitro* studies explored the obesogenic nature of 2-NAP. Findings revealed that 2-NAP accelerates adipose differentiation, promotes lipid accumulation, and perturbs high-energy metabolic processes. Our data analysis, alongside past studies, suggests that the immunomodulatory effects of PAHs can be attributed to direct exposure to specific LMW-PAHs.

Because obesity is a multifaceted and multifactorial disorder, it is necessary to identify the many other mechanisms LMW-PAHs could influence to contribute to the many metabolic dysfunctions associated with these compounds. By understanding how LMW PAHs contribute to obesity, it may be possible to develop strategies to prevent or reduce exposure to these chemicals and potentially reduce the risk of obesity and related health problems.

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APPENDICES

Appendix A – “Scientist” by Kal-El Tennant

