DIRECT EFFECTS OF PESTICIDES ON BROWN ADIPOSE TISSUE
IN VITRO EFFECTS OF PESTICIDES ON BROWN ADIPOSE TISSUE FUNCTION

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TITLE: In Vitro Effects of Pesticides on Brown Adipose Tissue Function

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ABSTRACT

Obesity and its associated comorbidities can occur when energy consumption exceeds energy expenditure. Brown adipose tissue (BAT) is an important contributor to energy expenditure as its mitochondria are rich in uncoupling protein 1 (UCP-1). UCP-1 uncouples oxidative phosphorylation from the generation of ATP to induce a futile cycle and consequently the generation of heat. Recent studies demonstrate that reduced BAT activity is correlated with many chronic metabolic diseases including obesity and type 2 diabetes, but the mechanisms mediating these effects are currently unclear. There are an increasing number of reports describing how pesticides, currently used to increase crop production, contribute to the development of metabolic syndrome including effects on adipogenesis, food intake, glucose and lipid homeostasis being reported. However, the effects of these compounds on BAT remain largely unknown. The objective of this study was to examine the direct effects of pesticides used to increase crop production on BAT function.

Nine pesticides and their primary metabolites were chosen to enter a 3-tiered screening process. Compounds were selected based on widespread usage and/or reports of metabolic perturbations in epidemiological studies, or animal experiments. Immortalized BAT cells derived from UCP-1 luciferase reporter mice, or wild-type FVB/N mice were treated with compounds at 1pM, 1nM, and 1μM doses. To elucidate the effects of selected pesticides on UCP-1 expression, UCP-1 promoter activity, mRNA and protein levels were quantified using a luciferase assay system, RT-qPCR, and western blotting respectively. The most consistent compound was further tested to determine its effects on BAT function by whole
cell respiration assay and mitochondrial enzyme activity assays (i.e., citrate synthase, and cytochrome c oxidase assay).

Of the 18 compounds that entered the primary screen, 8 compounds reduced UCP-1 promoter activity by >25%. RT-qPCR revealed that 5 compounds reduced UCP-1 mRNA levels at the same doses by 25% or more. Of these 5 remaining compounds, 3 also exhibited a reduction in UCP-1 protein content by >25%. Of these 3 compounds, chlorpyrifos was most consistent and further analyzed for its effects on BAT function. Chlorpyrifos reduced the expression of genes important for mitochondrial function, while also reducing maximal respiratory capacity and the activity of cytochrome c oxidase of treated brown adipocytes.

These data demonstrate that the commonly used pesticide, chlorpyrifos, directly suppresses UCP-1 expression and BAT function of cultured brown adipocytes from mice at concentrations as low as 1pM. Furthermore, these data also show that its action might be in part, by perturbations in complex IV (cytochrome c oxidase) of the electron transport chain of BAT mitochondria, in addition to the suppression of UCP-1.
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LIST OF ABBREVIATION

2,4-D – 2,4-Dichlorophenoxyacetic acid
3-PBA – 3-Phenoxybenzoic acid
AA – antibiotic, antimycotic
AC – adenyl cyclase
ACC – acetyl CoA carboxylase
AChE – acetylcholinesterase
AMPA – aminomethylphosphonic acid
AMPKα – adenosine monophosphate – activated protein kinase α
ANOVA – analysis of variance
Atf2 – activating transcription factor 2
ATGL – adipose triglyceride lipase
ATP – adenosine triphosphate
BAT – brown adipose tissue
β-HCH – β-hexachlorohexane
BMI – body mass index
BSA – bovine serum albumin
C/EBPa – CCAAT/enhancer-binding protein
cAMP – cyclic adenosine monophosphate
cGMP – cyclic guanosine monophosphate
Cox2 – cytochrome c oxidase subunit 2
Cox8b – cytochrome c oxidase subunit 8b
CPF – chlorpyrifos
Creb – cAMP response element binding protein
Cs – citrate synthase
DDE – dichlorodiphenyl dichloroethylene
DDT – dichlorodiphenyltrichloroethane
Dex – dexamethasone
DMEM – dublecco’s modified eagle’s media
DMSO – dimethyl sulfoxide
DTNB – 5,5-dithio-bis-(2-nitrobenzoic acid)
DTT – dithiothreitol
EDTA – ethylenediaminetetraacetic acid
EPA – Environmental Protection Agency
ER – endoplasmic reticulum
ETC – electron transport chain
FBS – fetal bovine serum
FCCP – carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone
Gw – GW6471
Hadh – hydroxyacyl-CoA dehydrogenase
HCB – hexachlorobenzene
HSL – hormone sensitive lipase
IBMX – 3-isobutyl-1-methylxanthine
Indo – indomethacin
Iso – isoproterenol
LXRα – liver X receptor α
MAPK – mitogen-activated protein kinase
MGL – monoacylglycerol lipase
MITC – methyl isothiocyanate
Myf5 – myogenic factor 5
NOAEL – no observed adverse effect level
NRF-1 – nuclear respiratory factor -1
NRF-2 – nuclear respiratory factor -2
pAMPKα - phosphoadenosine monophosphate – activated protein kinase α
PBS – phosphate buffered saline
PGC1α; Ppargc1α - peroxisome proliferator-activated receptor gamma coactivator 1-α
PKA – protein kinase A
PKB – protein kinase B
PKC – protein kinase C
PKG – protein kinase G
Pparα – peroxisome proliferator-activated receptor α
Pparγ – peroxisome proliferator-activated receptor γ
RIP 140 – receptor interacting protein 140
RLT – RNeasy lysis buffer
ROS – reactive oxygen species
RT-qPCR – real time-quantitative polymerase chain reaction
SEM – standard error of the mean
T3 – triiodothyronine
TBST – tris-buffered saline containing tween 20
TCP-y – 3,5,6-trichloro-2-pyridinol
Tfam – mitochondrial transcription factor A
TMBA1R3 – cell line derived from UCP-1 reporter mice
TMCON – cell line derived from wild-type FVB/N mice
UCP-1 – uncoupling protein 1
VDR – vitamin D receptor
VLDDL – very low-density lipoprotein
WAT – white adipose tissue
wBAT – brown in white adipose tissue; beige adipose tissue
CHAPTER 1: INTRODUCTION

1.1 Obesity

Obesity is a leading public health concern with worldwide prevalence of overweight (Body Mass Index, BMI ≥ 25 kg/m²) and obesity (BMI ≥ 30 kg/m²) rising 27% in the last three decades (1). It has been estimated that approximately 2.1 billion individuals are affected with overweight/obesity (1). In Canada, between the years 2007-2009 prevalence of obesity was estimated to be approximately 24.7% (2). Data from the Canadian Health Measures Survey (2007-2009), and Canadian Heart Health Surveys (1986-1992), revealed that over that 20-year period obesity prevalence increased in a statistically significant manner (2). The highest increases were seen in men between the ages of 60 and 74 (17 percentage points), and women between the ages of 20 and 39 (11.5 percentage points) (2). Consequently, scientists are turning towards investigating the causes of obesity and its associated co-morbidities to create better methods of combating metabolic disease.

One of the main causes of obesity is an imbalance in energy where energy intake exceeds energy expenditure, with sedentary life styles and a society that promotes overeating, contributing to reduced caloric use and increased daily caloric intake (3,1). However, this does not explain how in a shared environment, only a subset of individuals develop obesity (1). Now, scientists have revealed heritability estimates of obesity range between 0.25-0.90, suggesting that obesity risk may be a result of inherited factors (1). However, scientists have also revealed genetic variants that can protect against obesity (1).
Additionally, the human genome has not changed drastically over the last few years indicating if obesity was largely due to genetic variants, these variants would most likely be fixed in humans as they are advantageous to survival, and consequently obesity would be the norm (1). This is not the case. A study by Keith, et al., in 2006 revealed ten other factors that may be contributing to the obesity epidemic (3). Other factors that were identified included drug-induced weight gain, and exposure to endocrine disruptors such as pesticides, plastics, plasticizers, solvents or lubricants and their by-products (3, 4). Obesity is of great concern to the medical community. Not only are there several causes but they are also associated with several co-morbidities including: type-2 diabetes, osteoarthritis, depression, hypertension, cardiovascular disease, and cancer (1).

One of the most common co-morbidities associated with obesity is type-2 diabetes. Current estimates suggest approximately 171 million individuals have type-2 diabetes with this number expected to rise to 366 million by the year 2030 (5). The association that exists between obesity, insulin resistance and type-2 diabetes is recognized to be very complicated and involves several tissues including: the brain, liver, muscle, fat and pancreas (5). A review by Kahn, S., et al, explains the critical role of insulin release to this association (5). Impaired insulin secretion causes decreased insulin signaling to the hypothalamus, which leads to increased food intake/weight gain, hepatic glucose production, lipolysis in adipocytes and reduced efficiency of glucose uptake by muscles (5). All these events lead to increased levels of non-esterified fatty acids and glucose in the blood (5). Together, increased non-esterified fatty acids, glucose in the blood and
weight gain suppresses the β-cells adaptive response to insulin resistance and further adversely effects β-cell health and insulin action (5). This highlights the intricacy of normal metabolic function and how several tissues play an important role in maintaining metabolic homeostasis.

In the past, adipocytes were considered to play a passive role in the development of obesity and its associated co-morbidities. Now we know that adipocytes are not only a storage facility for fat but also critical components of metabolic control and endocrine organs that partake in both good and bad effects (6). The importance of adipocytes was highlighted in genetically altered mouse studies where mice with no fat tissue (i.e., lipoatrophic) showed similar characteristics to humans with severe lipoatrophic diabetes (i.e., insulin resistance, hyperglycemia, hyperlipidemia, and fatty livers) (6). These studies also showed that transplantation of adipose tissue from healthy mice into these lipoatrophic mice caused improved muscle insulin sensitivity, decreased serum triacylglycerols, hepatic gluconeogenesis, and amounts of fat depots in muscle and liver tissue (6). Adipose tissue has the capacity to change dramatically in response to nutritional status, with changes in, size, function, inflammatory state, and whole-body distribution (7). At a cellular level it changes its extracellular matrix, composition, vascularization, adipocyte size and number, oxidative stress level, adipokine secretory profile, inflammatory state and infiltration by immune cells (7). Obesity-induced adipose tissue expansion occurs via adipocyte hypertrophy/hyperplasia, which also causes a shift in the types of adipokines that are secreted by the tissue (7). In obesity, adipose tissue
becomes dysfunctional and fails to appropriately expand to store surplus energy (7). This leads to ectopic fat deposition in other tissues that regulate metabolic homeostasis (7). This causes elevated pro-inflammatory factors, and a parallel reduction in anti-inflammatory factors that may consequently lead to insulin resistance, initiating the progression to prediabetes and subsequently type-2 diabetes (7).

1.2 Brown Adipose Tissue (BAT) Function and Metabolic Disease

Mammals have 3 distinct types of adipose tissue: white adipose tissue (WAT), brown adipose tissue (BAT) and more recently discovered, beige adipose tissue (wBAT) (brown in white) (8). WAT can be separated into visceral fat; which includes mesenteric gonadal, epicardial, retroperitoneal, omental, and peri-renal; and subcutaneous WAT, which is often believed to be protective against obese phenotype (9). Active BAT can be located in cervical, supraclavicular, axillary, mediastinal, paraspinal, and abdominal anatomical regions; with the majority of BAT concentrated to the 4 upper torso depots (10,11). wBAT can be found within WAT deposits, most commonly in subcutaneous WAT, in response to classic BAT activators (12). WAT and BAT differ in origin, morphology, mitochondrial content, and gene expression of markers important to thermogenesis (9).

Mesenchymal derived WAT contains large unilocular lipid droplets and low cytoplasmic volume (9). The main function of WAT is to serve in endocrine signaling and to store energy in the form of triglycerides (9). WAT’s low mitochondrial content and reduced oxidative rates are what allow these tissues to store high levels of energy (13). BAT by
contrast, originate from myogenic factor 5 (Myf5)- expressing progenitor populations, BAT cells contain multilocular lipid droplets, and contain high levels of mitochondria giving them their characteristic brown colour (8-26). BAT is responsible for non-shivering thermogenesis in response to food, exercise and cold exposure (8, 13, 24). These tissues function to maintain temperature homeostasis by producing heat via uncoupling protein 1 (UCP-1) (8-26). UCP-1 generates heat by uncoupling ATP synthase from the electron transport chain (8). wBAT are UCP-1 expressing brown-like adipocytes that develop in WAT in response to BAT activators (8). The origin of wBAT still remains unclear although studies have suggested that they derive from a different lineage than BAT and express unique signatures, while others suggest that β-adrenoreceptor agonist stimulated mature WAT cells are the origins of these cells (i.e., transdifferentiation) (12, 20). Classic BAT activators include: cold exposure, feeding, exercise or adrenergic stimulation (norepinephrine) (8, 20, 27). Adipose tissue’s role in the regulation of metabolism and energy intake can also be attributed to its ability to secrete large numbers of proteins termed adipokines that act in an autocrine, paracrine and endocrine fashion (6). All 3 types of adipose tissue play an important role in maintaining normal metabolic function and whole-body metabolic homeostasis.

In humans, BAT was believed to be present solely in infants and children. Its presence in adults was believed to be non-existent and physiologically irrelevant (14). However, in 2009 several studies demonstrated that active BAT can be found in adult humans and if present, is inversely correlated to age and body mass (14, 87, 103 - 106). Most recent
studies have shown that obese individuals have impaired BAT function, demonstrating an association between BAT and the obese phenotype (14, 15). Although interesting, it is still unclear whether these observations are the cause or a consequence of obesity (14, 15). Nevertheless, the association between BAT and the obese phenotype has been of great interest to investigators in looking at the potential of using BAT to combat obesity. Obesity can result from an imbalance in energy where energy intake exceeds energy expenditure (14). When activated, BAT consumes more glucose per gram than any other tissue in the body (15). Animal studies have shown that pharmacological and nutritional activation of BAT can cause a resistance to obesity, and improve glucose tolerance and insulin sensitivity (8, 18). Mice lacking functional BAT have been shown to gain more weight than wild-type mice (28). These findings support the hypothesis that BAT is important for body weight homeostasis.

Not only does BAT play an important role in body weight homeostasis, studies have also demonstrated its role in glucose homeostasis (29-31). For example, Stanford, K. et al, in 2013, showed that increasing BAT mass by transplantation from age and sex matched donor mice improved glucose homeostasis and insulin sensitivity (30). This study also reported that by 8-12 weeks post transplantation mice showed improved glucose tolerance, increased insulin sensitivity, increased insulin stimulated glucose uptake of BAT, WAT and heart muscle, lowered body weight, fat mass, and total reversal of high fat-induced insulin resistance. Furthermore, they demonstrated that these effects were further improved with increased mass of transplanted BAT. Another study in mice
demonstrates that SIRT1 (an NAD\(^+\)-dependent protein deacetylase) improves glucose homeostasis by enhancing BAT function by potentiating response to \(\beta_3\)-adrenergic stimuli (31). Mice with increased BAT activity exhibited enhanced insulin sensitivity, effects that were reversed when BAT was inactivated upon exposure to thermoneutral conditions (31). These studies demonstrate a role that BAT plays in glucose homeostasis and how BAT function may be a target to exploit for the development of therapeutics aimed to improve glycemic control.

1.2.1 Regulation of BAT function

BAT activation occurs via the \(\beta_3\)-adrenergic/cAMP signaling pathway (8). Increased stimulation of the sympathetic nervous system results in the release of norepinephrine (8, 13, 16). Norepinephrine binds to \(\beta_3\)-adrenergic receptors which are coupled to stimulatory G-proteins that activate adenylyl cyclase (AC) (8,13,16,20,22,26). AC catalyzes the conversion of adenosine triphosphate (ATP) to cyclic adenosine monophosphate (cAMP) (13). Increased concentrations of cAMP activate protein kinase A (PKA) (8,13,16,20,22,26). PKA or cGMP-dependent protein kinase (PKG) in turn activates downstream pathways that lead to thermogenesis and lipolysis (8,13,16,20,22, 26). PKG is activated by cGMP release as a result of natriuretic peptide signaling (8). First, PKA/PKG activates p38 mitogen activated protein kinase (MAPK) and cAMP response element binding protein (Creb) (8). p38 MAPK induces the activation of activating transcription factor 2 (Atf2) and peroxisome proliferator-activated receptor gamma coactivator 1 alpha (PGC1\(\alpha\)) (8). Atf2 and PGC1\(\alpha\) induce the transcription of
thermogenic genes such as UCP-1 through interactions with DNA via peroxisome proliferator-activated receptor gamma (Ppar-γ), peroxisome proliferator-activated receptor alpha (Pparα), retinoid x, and thyroid receptors (8). PKA/PKG activates several lipases such as adipose tissue triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoacylglycerol lipase (MGL) (8,13). These lipases release free fatty acids and glycerol which in turn, are transported into the mitochondria and are used to activate UCP-1 and fuel thermogenesis (8,13,32). Phospholipases in the inner mitochondrial membrane have also been show to release free fatty acids that directly activate UCP-1 (32). Active UCP-1 then uncouples oxidative phosphorylation from ATP-synthase resulting in a futile cycle that results in the generation of heat from the combustion of available substrates (8). Mitochondrial proton-leak makes up approximately 20% of whole-body energy expenditure (18).
Figure 1. Induction of BAT thermogenesis via β-adrenergic/cAMP signaling cascade as depicted in Harms and Seale, (2013).

Although the signaling pathways leading to BAT activation have been well characterized there is limited information available regarding factors which contribute to decreased BAT functional capacity. This line of inquiry has recently gained increased attention as scientists attempt to understand how impaired BAT function could contribute to obesity and its associated co-morbidities.

1.3 Pesticides and Metabolic disease

Excess caloric intake and inactivity are thought to be the main contributors to the obesity epidemic, an increasing problem in today’s society. Now, there is evidence pointing
towards chemicals in the environment playing a role in the development of obesity and its associated comorbidities. The role of environmental chemicals in the development of obesity and diabetes is a relatively new area of study but there is accumulating evidence that these exposures play a key role in the development of chronic metabolic diseases (33). One class of chemicals, which have been widely studied for their link to metabolic diseases, are compounds used on agricultural crops to combat pests (i.e., pesticides) (33 - 35). Pesticide demand continues to grow due to their ability to increase global agricultural productivity, reduce insect-borne endemic diseases, and protect/restore plantations, forests, harvested wood products, homes, and fibers (36).

Pesticides include: insecticides, fungicides, herbicides and compounds that can be classified under all categories, such as soil fumigants (37). Insecticides are divided into five major classes divided by chemical structure and mode of action (38). These include: organochlorines, organophosphates, carbamates, pyrethroids and neonicotinoids (38). Organophosphorus insecticides are acetylcholinesterase (AChE) inhibitors that cause hyperstimulation of cholinergic nerves (38,39). Pyrethroids are structural analogs of naturally occurring insecticides pyrethrin, found in Chrysanthemum flower heads (38,40). Pyrethroid’s toxic effects are by over exciting the neurons causing constant firing of action potentials (38,40). Neonicotinoids, similar to nicotine, target nicotinic acetylcholine receptors, which are ion channels that play an important role in nerve signaling (41). Herbicides, another class of commonly used pesticides, are divided into two categories: inhibitors of photosynthesis, and non-inhibitors (42). Inhibitors of
photosynthesis comprise of more than half of the market these include triazines, such as atrazine (42). Several other herbicides are in the class of non-inhibitors. One example is 2,4-dichlorophenoxyacetic acid (2,4-D), a synthetic auxin (plant hormone) that causes uncontrolled plant growth that leads to plant death, is one of the oldest herbicides on the market (44). Although a new compound, glyphosate, has seen a significant increase in usage as it has been identified as toxicologically and environmentally safe (43,44). Glyphosate imparts its effects by inhibiting an enzyme central to the shikimate pathway (important for biosynthesis of folates and aromatic amino acids in bacteria, fungi, etc.) (43). A commonly used soil fumigant and wood preservative is metam sodium (45). The U.S Environmental Protection Agency (EPA) named metam sodium the third most commonly used pesticide (46). This compound interferes with respiratory enzymes by chelating metal ions (45). As demand from the agricultural industry for pesticides usage to increase crop production rises, it is important that scientists continue to investigate off-target effects of currently used pesticides that may contribute to chronic diseases.

1.3.1 Human Exposure

In Canada, there are over 7000 pesticide products that are currently registered for use, mainly in agriculture to increase food production (47). Human exposure to pesticides is inevitable. Not only does the high use of these compounds pose a risk to the individuals who work in the agricultural industry, these chemicals also have the ability to enter our environment (air, soil, water, human and animal tissue) and accumulate for long periods of time (37, 47). Pesticide exposure can occur through several routes: skin (dermal,
primary exposure route), eyes, inhalation or ingestion (37). Individuals at high risk of pesticide exposure are individuals who handle, transport, deliver, or apply pesticides (occupational exposure); and individuals who live in areas where high pesticide residues can be detected. The majority of the population are exposed to pesticides via ingestion of foods that contain pesticide residues that have accumulated on the surface during application (48).

Pesticides themselves or their metabolites have been detected in human blood, urine, fat, serum, and breast milk samples (37). The distribution of these compounds is very dependent on their chemical structure and varies from class to class. For example, 2,4-D, a herbicide that is slightly water soluble, accumulates primarily in the lung, heart, liver, spleen, and kidney (44), whereas choryprifos, a highly lipophilic insecticide, primarily accumulates in fat and brain tissue (39,49). Despite being designed to target pests, these compounds are not completely selective (37,50). For this reason, there is increased concern regarding the health effects exposures to these mixtures of pesticides as exposure is continuous and the distinct components of the mixture are often unknown (37,50).

1.3.2 Epidemiological Studies

Pesticides have been investigated for several years to assess their risk on human health. The strongest association reported has been a link between pesticide exposure and an increased incidence of cancer (Figure 2) (48,51). Additionally, several studies have identified an association between pesticide exposure and reproduction/developmental
More recently there has been considerable interest in the role of pesticide exposures in the development of metabolic diseases such as obesity and type-2 diabetes (51). Obesity and its associated co-morbidities, such as type-2 diabetes, continues to be a growing epidemic worldwide, with prevalence growing exponentially since the 1980s (52). Today, several chemicals in the environment have been coined “obesogens” (53). Obesogens are compounds in the environment that promote adiposity by altering fat cell development, increasing energy storage in adipocytes, and altering neuroendocrine control of appetite and satiety (53).

Figure 2. Schematic diagram showing the weight of evidence on toxicities of pesticides as reported in Mostafalou, and Abdollahi, (2017). ND; not determined.

However, although pesticides have been shown to impact molecular pathways important for the development of obesity and diabetes, there are few epidemiological studies investigating the association between pesticides and obesity (48). An epidemiological study looking at 7 cross-sectional and a cohort analyses demonstrated that an increased BMI was associated with exposure to pesticides such as Dichlorodiphenyldichloroethylene (DDE), Dichlorodiphenyltrichloroethane (DDT),
hexachlorobenzene (HCB), β-hexachlorohexane (β-HCH), trans-nonachlor, and oxychlordane (48). Other studies investigating the link between pyrethroids and obesity demonstrated an association between exposure of these compounds and disrupted lipid metabolism - reporting elevated triglycerides, phospholipids and very low-density lipoprotein cholesterol (VLDL) (38).

A systematic review investigating the potential contribution of insecticide exposure on the development of obesity and types-2 diabetes discovered that, organophosphorus and organochlorine insecticides are the most investigated classes, and pyrethroids, carbamates, and neonicotinoids have also been studied to a lesser extent (38). In this review, they revealed that exposure to organochlorine, organophosphorus, and carbamate insecticides increases risk of diabetes when considering elevated blood glucose and insulin resistance as markers of diabetes (38). The National Health and Nutrition Examination Survey examining the health and nutritional status of adults and children, reported that there is a significantly increased risk of diabetes and insulin resistance with pesticide exposure (48). A meta-analysis of observational studies analyzing the risk of diabetes from pesticide exposure reported a risk estimate of 1.58 (95% CI: 1.32–1.90, p = 1.21 × 10⁻⁶) with the highest association seen with organochlorine exposure (i.e., DDE, heptachlor, HCB, DDT, trans-nonachlor, chlordane) (48). An investigation looking at the risk of diabetes from organophosphorus insecticide exposure reported a significant association between organophosphorus insecticides and hyperglycemia (48). A cohort study investigating the association between lifetime exposure to pesticides and diabetes,
reported a significant increased risk of diabetes in those exposed to aldrin, chlordane, heptachlor (organochlorines); dichlorvos, trichlorfon (organophosphates); alachlor and cyanazine (herbicides) (48). A study investigating the effects of occupational exposure of pesticides reported an increased risk of diabetes in applicators that used organochlorine or organophosphorus insecticides, some of which followed a dose-dependent effect (54). Furthermore, an investigation on the risk associated with pregnant wives of pest applicators exposed to pesticides demonstrated an increased risk of gestational diabetes mellitus, and pregnancy-induced hypertension (35,38,102). Additionally, exposure to pyrethroid mixtures significantly increased the risk of diabetes among factory workers in China (38). Investigating why these associations are being seen is important and often studied in animal and cell culture models.

### 1.3.3 In Vitro and In Vivo Studies

Several studies investigated the effects of pesticides in vivo and in vitro on metabolic function. Numerous studies have reported that certain compounds in all major classes of insecticides (i.e., organochlorine, organophosphorus, carbamate, pyrethroids, neonicotinoids) cause elevated blood glucose, although this is dependent on dose, route of exposure, animal species, and exposure duration (38). For example, 50mg/kg DTT (organochlorine insecticide) exposure in male mice was able to reduce both glucose tolerance and insulin secretion (55). These effects persisted for a week post single dose administration (55). A study by Panahi, et al., demonstrated that malathion, an organophosphorus insecticide, induces hyperglycemia and increase insulin levels in both
acute and sub chronic exposure (56). Oral exposure (below the No Observable Adverse Effect Level; NOAEL) to permethrin (pyrethroid) or imidacloprid (neonicotinoid) potentiated insulin resistance when mice were on high-fat diet (38). Although there are studies linking compounds from all major classes with either elevated blood glucose or decreased insulin sensitivity, insecticides classified as organochlorine or organophosphorus compounds have been shown to be the most commonly identified as potential disruptors of glycemic control (38).

A study of 5 groups of Wistar rats exposed to 2 doses of chlorpyrifos (organophosphorus insecticide) from gestation until weaning revealed that chlorpyrifos caused increased birth body weight, hyperglycemia and decreased insulin levels at the high dose exposure levels (57). After low dose exposure, rats exhibited hyperinsulinemia as a result of decreased insulin receptor β in liver. A similar study by Fang B. et al., where normal or high fat diet fed rats were exposed to 0.3 or 3.0 mg/kg body weight/day chlorpyrifos demonstrated diet specific effects on metabolism and gut microbiota (58). This study demonstrated that there was an increase in weight gain in normal fat fed mice, and although there were no changes in food intake or weight gain in high fat fed rats in this study as shown in previous studies, they did demonstrate that chlorpyrifos induced changes in gut microbiome communities. They also showed that changes in the gut microbiome communities of exposed rats were exacerbated by high-fat diet. There is controversy in literature on the effects of pesticide on weight gain in animal studies. This is often because there are several factors that affect the outcome of weight gain, one of particular
importance is dose. Often high dose exposure studies are toxic and therefore cause weight loss rather than weight gain. There is evidence from rodent studies that chronic low dose exposure to insecticides and herbicides results in significant weight gain (38,59). For this reason, low/biologically relevant dose studies are very important when studying the obesogenic effects of pesticides used in agriculture.

*In vitro* studies have also helped to elucidate the potential of pesticides as obesogens. For example, a study using 3T3-L1 adipocytes demonstrated that DDT, DDE (orchanochlorine); imidacloprid (neonicotinoid); and permethrin (pyrethroid) potentiated adipogenesis (38). Another study using 3T3-L1 cells showed that organochlorine pesticides caused down-regulation of insulin induced gene-1 and Lpin-1, key regulators of lipid metabolism (38). Furthermore, 3T3-L1 cells treated with DDE increased basal free fatty acid uptake and increased the release of adiponectin, leptin and resistin (38). Elevation of these hormones are associated with obesity and type-2 diabetes (9, 38). A study using C2C12 muscle cells demonstrated that permethrin (pyrethroid) and imidacloprid (neonicotinoids) induce insulin resistance via protein kinase B (PKB) signaling (38). Taken together these studies support the hypothesis that pesticides can act to perturb metabolic homeostasis although the underlying mechanisms have not yet been fully elucidated. Notably, to date, there has been little attention to the impact of these compounds on BAT activity which may be an alternative mechanism to explain their adverse impact on weight gain and glucose homeostasis.
1.4 Pesticide-induced mitochondrial dysfunction

The mechanisms in which pesticides impart their effects on metabolic function remain relatively unknown and depend on doses, duration/route of exposure, species and pesticide class. However, inhibition of mitochondrial function may represent a unifying hypothesis for pesticide impact on metabolism. Indeed, there are numerous studies showing that pesticides can impact mitochondrial function of multiple tissue types (33,34,59 - 70,80).

Mitochondria are the main source of ATP and reactive oxygen species (ROS) and play an important role in metabolic cell signaling and cellular energy homeostasis (71,72). Consequently, mitochondrial dysfunction is the most studied perturbation that occurs in metabolic disease (63). Mitochondrial dysfunction is a reduction in mitochondrial oxidation of substrates, including lipids and carbohydrates, resulting in decreased oxidative phosphorylation (mitochondrial respiration) (71). Total cellular content and function of mitochondria can be assessed by a variety of measurements including: the expression of mitochondrial markers, enzymes that make up the electron transport chain (ETC) (complex I-V), the activity of mitochondrial enzymes, morphology of the mitochondria, substrate oxidation and ROS production (71). Pesticides are known effectors of mitochondria. Most of these compounds either directly or indirectly affect oxidative phosphorylation. Changes in oxidative phosphorylation occur via disruption of processes that provide substrates for mitochondria or inhibiting the mitochondrial respiratory chain itself. Many of these compounds inhibit structures very similar to
mitochondria such as thylakoid membrane in the chloroplast of plants (34,60,68). For example, organophosphorus insecticides have been shown to reduce expression/activity of several components of the mitochondrial electron transport chain (ETC) (64). These insecticides have been demonstrated to increase mitochondrial calcium uptake resulting in inhibition of complex I and II of the ETC and decreased complex IV electron transfer in a human neuroblastoma cell line (73). Furthermore, pyrethroid insecticides have also been shown to inhibit mitochondrial ETC enzyme activity of rat liver mitochondria (60). The herbicide atrazine has been shown to inhibit mitochondrial capacity in liver and muscle tissue of exposed mice by reducing beta-oxidation of fatty acids, decreasing activity of complexes I and III, and disrupting mitochondrial morphology via swelling and disrupting cristae (34, 59, 63). The herbicide 2,4-D has been shown to inhibit complexes II and III and disrupt mitochondrial membrane potential via uncoupling in rat liver mitochondria (60). In addition to reduced mitochondrial respiratory function, disruption of the mitochondria can cause increased oxidative stress (4). Oxidative stress can induce inflammatory responses that are often associated with obesity and other metabolic diseases (4). Organophosphorus insecticides have been shown to increase ROS and lipid peroxidation in both human and animal studies (66). Pyrethroids have been associated with oxidative stress in several tissue types such as liver, kidney, and brain (66). These and many other studies show the negative effects of pesticides on mitochondrial function and, as the mitochondria play a central role in BAT thermogenesis, may represent a mechanism for their potential for disruption of BAT function (4,8,34, 59,60, 63,64, 66, 68, 71 - 73).
CHAPTER 2: HYPOTHESIS AND OBJECTIVES

2.1 Rationale and Hypothesis

*Rationale:*

Obesity and its associated comorbidities can result from an imbalance of energy, where energy consumption exceeds energy expenditure. Studies are now demonstrating the importance of BAT not only in childhood but also in adult metabolism (14). BAT is important for both basal and inducible energy expenditure, as its mitochondria are rich in uncoupling protein 1 (UCP-1) (14,15,74). UCP-1 allows oxidative phosphorylation to be uncoupled from ATP synthesis resulting in a futile cycle that ultimately produces heat from the combustion of available substrates (8,74). BAT has now been shown to affect whole body metabolism, insulin sensitivity, and susceptibility to weight gain (14). A decrease in BAT function can lead to decreased energy expenditure, which can contribute to metabolic disease. Studies are now showing that pesticides, used to increase crop production, have been linked to chronic diseases such as obesity and its associated comorbidities (52,54,63,75). In Canada alone, 7000 pesticide products are registered for use and persist in the environment (47). Pesticides have been linked to disruption of glucose and lipid metabolism, type-2 diabetes, insulin resistance, increased food intake, mitochondrial dysfunction, and oxidative stress (63,76,77). Although studies continue to show the link between pesticides and metabolic syndrome the effects of these compounds on BAT remain unknown.
Hypothesis:

Low dose exposure to pesticides will decrease brown adipocyte function and have the potential to contribute to metabolic disease.

2.2 Objectives

The specific aims of my M.Sc. project are:

1) To examine the direct effects of pesticides on BAT using immortalized BAT cells derived from luciferase reporter mice, or wild-type FVB/N mice.

2) To determine the mechanism(s) underlying the effect of our “hit” compounds on BAT function
CHAPTER 3: MATERIALS AND METHODS

3.1 Cell culture, murine brown adipocyte differentiation and treatments

Cell Lines

Two immortalized brown pre-adipocyte cell lines were used in this study. Cell lines were created by Ph.D candidate Alex Green from the UCP-1-Luc2-TdTomato reporter mouse, also known as the “ThermoMouse” (78) using techniques described by Uldry et al. (2006) (101). Briefly, BAT tissue was harvested from 4-day old pups, and subsequently digested with collagenase II, filtered, and plated. Isolated cells were then infected with a retrovirus created by transfecting Phoenix-ECO cells with a pBABE-SV40 plasmid from Addgene. Cells positive for SV40 were selected for 7-days using 2 µg/ml puromycin antibiotic, and underwent a 14-day treatment with the antibiotic ciproflaxin (10 µg/ml) in order to eliminate any mycoplasma contamination in the cells. Cells positive for UCP-1 Luc2-TdTomato (named TMBA5R3) were used for all luciferase assays to quantify UCP-1 promoter activity. Wildtype cells derived from FVB/N mice (named TMCONs) were used for the remainder of experiments to avoid any unknown confounding effects of the transgene insertion.

Maintenance and Differentiation

Immortalized brown pre-adipocytes (TMBA5R3 and TMCON) were grown in Dulbecco’s Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS) (Life Technologies) and 1% antibiotic and antimycotic (AA) (Life Technologies) at 37°C in a humidified atmosphere of 95% air
and 5% CO₂. Cells were grown in 175cm² (ThermoScientific, Rochester, NY, USA) flasks and passaged once 70-80% confluence was reached. For experiments cells were counted using a hemocytometer and plated at 10,000 cells/cm² into 75cm² flasks, 24 or 12-well plates (ThermoScientific). 48 hours post-plating, cells reached 90-95% confluency (Day 0) and were induced to differentiate for 9 days. On day 0, cells were treated with an induction media consisting of DMEM supplemented with 10% FBS, 1% AA, 20nM insulin (Life Technologies), 1nM triiodothyronine (T3) (Sigma-Aldrich, St. Louis, MO, USA), 0.5μM dexamethasone (Dex) (Sigma-Aldrich), 0.5mM 3-isobutyl-1-methylxanthine (IBMX) (Sigma-Aldrich), and 125μM indomethacin (Indo) (Sigma-Aldrich) for 48 hours. On day 2 of differentiation, cell media was switched to maturation media consisting of DMEM supplemented with 10% FBS, 1% AA, 20nM insulin, and 1nM T3. Maturation media was refreshed every 48 hours for the remainder of the differentiation period. On day 9 of differentiation cells were deemed mature brown adipocytes and were ready for treatment with test compounds (refer to figure 3).

**Figure 3. In vitro** protocol for brown adipocyte differentiation and treatments.
Treatments

On day 9 of differentiation cells were treated with test compounds or controls. Controls consisted of vehicle (≤0.1% dimethyl sulfoxide; DMSO) (Sigma-Aldrich), 10μM isoproterenol (Iso)(Sigma-Aldrich) a β-agonist which activates BAT, and 9μM GW6471(Gw) (Cayman Chemical Co., Ann Arbor, MI, USA) - a PPARα antagonist. Following differentiation, cells were treated for 16-hours for UCP-1 promoter stimulation analyses, 4 - 4.5 hours for mRNA analysis, and 24-hours or 6-days for western blot, whole-cell respiration, citrate synthase, and cytochrome c oxidase assays. For treatments longer than 24-hrs, media was replenished every 48hrs. In order to determine if the test compounds could inhibit adrenergic stimulated BAT function, cells were co-treated with one of our test compounds chlorpyrifos ± Iso. In this experiment, cells were plated into 24-well plates and differentiated as previously outlined. Following differentiation, cells were either treated with chlorpyrifos and isoproterenol simultaneously for 6 days for protein analysis; or pretreated with chlorpyrifos for 30min prior to adding isoproterenol for 4 hours for mRNA expression data. After treatment, cells were washed briefly with 5mL Phosphate buffered saline (PBS) (Life Technologies), snap frozen using liquid nitrogen and stored at -80°C until further analysis.

3.2 Compound Selection

Nine pesticides and nine of their primary metabolites were selected to enter a three-tiered screening process in order to examine the direct effects of these chemicals on BAT
function (refer to Table 1). Compounds that reduced UCP-1 promoter (tier 1), mRNA (tier 2), and protein (tier 3) levels by greater than 25% at 1pM and/or 1nM moved forward in the screening process. Pesticides were chosen in collaboration with the National Institute of Environmental Health (United States) and Health Canada or were selected from the ToxCast® chemical library http://www.epa.gov/ncct/toxcast/. These compounds were selected based on widespread use in Canadian agriculture, compounds that have been previously shown to alter metabolic function and compounds of interest to our collaborators (Drs. Kristina Thayer and Mike Wade). All compounds were dissolved in DMSO to create 1mM stock solutions. The stock solution was then serially diluted in DMSO to create 1μM, and 1nM stock solutions of each compound. 1mM stocks were then diluted to create 1μM working test solutions. 1μM stocks were diluted to create 1nM working test solutions. Finally, 1nM stocks were used to create 1pM working test solutions. Cells were also treated with vehicle (control; ≤ 0.1% DMSO), Iso (10 μM; positive control) and Gw (9μM; negative control) using 10 mM (Iso), and 9mM (Gw) stocks which were diluted to create working test solutions.
Table 1. Pesticides Chosen for Analyses. Chemicals were purchased from Toronto Research Chemicals Inc., Toronto, ON, CA.

<table>
<thead>
<tr>
<th>Compound (CASRN)</th>
<th>Metabolites</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-D (94-75-7)</td>
<td>Excreted mostly unchanged</td>
</tr>
<tr>
<td>Atrazine (1912-24-9)</td>
<td>Diaminochlorotriazine (3397-62-4)</td>
</tr>
<tr>
<td></td>
<td>Desethylatrazine (6190-65-4)</td>
</tr>
<tr>
<td></td>
<td>Atrazine mercapturate (138722-96-0)</td>
</tr>
<tr>
<td>Chlorpyrifos (2921-88-2)</td>
<td>Chlorpyrifos Oxon (5598-15-2)</td>
</tr>
<tr>
<td></td>
<td>3,5,6-trichloro-2-pyridinol (TCP-y) (6515-38-4)</td>
</tr>
<tr>
<td>Deltamethrin (52918-63-5)</td>
<td>3-Phenoxybenzoic Acid (3-PBA) (3739-38-6)</td>
</tr>
<tr>
<td>Glyphosate (1071-83-6)</td>
<td>Aminomethylphosphonic acid (AMPA) (1066-51-9)</td>
</tr>
<tr>
<td>Imidacloprid (138261-41-3)</td>
<td>6-chloronicotinic acid (5326-23-8)</td>
</tr>
<tr>
<td>Metam Sodium (137-42-8)</td>
<td>Methylisothiocyanate (MITC) (556-61-6)</td>
</tr>
<tr>
<td>Permethrin (52645-53-1)</td>
<td>3-Phenoxybenzoic Acid (3-PBA)</td>
</tr>
<tr>
<td>S-metolachlor (87392-12-9)</td>
<td>Not appreciably metabolized</td>
</tr>
</tbody>
</table>

3.3 Luciferase assay: UCP-1 promoter analysis

TMBA5R3 brown adipocytes were differentiated in 24-well plates and treated on day 9 of differentiation with test compounds for 16hrs. Cell lysates were collected using Promega
passive lysis buffer (Promega, Madison, WI, USA) (75μL/ well) and transferred into 1.5ml centrifuge tubes. In order to further lyse cells, lysates were then flash frozen using liquid nitrogen and allowed to thaw on ice before being centrifuged at 16,000 x g for 10 minutes at 4°C. Supernatant was collected and 20μl was used to assay each sample in duplicate in white 96-well plates (Corning Inc., Kennebunk, ME, USA). 50μl of active luciferase assay reagent (Luciferase Assay System E1500, Promega) was then added to 2 columns (16 wells) of the 96-well plate at a time and immediately read for luminescence on SpectraMax M5 (Molecular Devices, San Jose, California, USA) plate reader. All samples in 96-well plates were read on plate reader 2 columns at a time to ensure that each reaction was read within 1 minute of administration of active assay reagent.

3.4 RNA Analysis

TMCON brown pre-adipocytes were differentiated in 24-well plates and treated with test compounds on day 9 of differentiation. Cells were treated for 4 hours in Krebs-Ringer Bicarbonate Buffer supplemented (Sigma-Aldrich) with 10mM HEPES (Janssen Pharmaceuticals, 3a2440 Geel, Belgium), 1mM CaCl₂, and 1% fatty-acid free Bovine Serum Albumin (BSA) (EquiTech Bio Inc., Texas, USA) at pH 7.4.

RNA Extraction

Cell lysates were collected by administering 175μL per well of RNeasy Lysis Buffer (RLT) (Qiagen, Hilden, Germany) supplemented with 143mM β-mercaptoethanol (Sigma-Aldrich) and vortexing until cells lifted from plates. Once cells were lifted from
plates, 175μL of 70% ethanol is added to each well and lysates are transferred to a High Pure Filter tube from Roche RNA Isolation Kit (Roche Diagnostics, Mannheim, Germany). RNA isolation was done as outlined by manufacturers specifications.

**Complimentary DNA (cDNA) Synthesis**

Once RNA was extracted, nanophotometer (Implen Inc.; Westlake Village, CA) was used to measure RNA concentration of each sample. A total of 1μg of cDNA was made using SuperScript III Reverse Transcriptase (Life Technologies) and Biometra Thermocycler (Montreal Biotech Inc., Dorval, QC, CA) as per manufacturers specifications. cDNA was then diluted 1:10 and stored at -20°C until further use.

**Real-Time Quantitative Polymerase Chain Reaction (RT-qPCR)**

Real-time quantitative PCR was performed using TaqMan® Gene Expression Assays (Life Technologies) and Rotor-Gene 6000 (Corbett Research; Mortlake, Australia). Refer to Table 2 for information on TaqMan probes. Each sample was run in duplicate and analyzed in a two-step protocol as follows: polymerase activation (95°C for 10 min), 40 cycles of: 1) denaturing (95°C for 10 sec) and 2) annealing (60°C for 45 sec). Relative mRNA levels were quantified using the $2^{\Delta\Delta CT}$ formula and corrected to the expression of *Ppia*. 
**Table 2**: TaqMan Probes. All probes purchased from Life Technologies.

<table>
<thead>
<tr>
<th>Probe</th>
<th>Reference Sequence</th>
<th>Assay ID</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ucp-1</em></td>
<td>NM_009463.3</td>
<td>Mm01244861_m1</td>
</tr>
<tr>
<td><em>Cox8b</em></td>
<td>NM_007751.3</td>
<td>Mm00432648_m1</td>
</tr>
<tr>
<td><em>Cox2</em></td>
<td>NC_005089_COX2.0</td>
<td>Mm03294838_g1</td>
</tr>
<tr>
<td><em>Cs</em></td>
<td>NM_026444.3</td>
<td>Mm00466043_m1</td>
</tr>
<tr>
<td><em>Hadh</em></td>
<td>NM_008212.4</td>
<td>Mm00492535_m1</td>
</tr>
<tr>
<td><em>Ppargc1α</em></td>
<td>NM_008904.2</td>
<td>Mm00447183_m1</td>
</tr>
<tr>
<td><em>Ppia</em></td>
<td>NM_008907.1</td>
<td>Mm02342430_g1</td>
</tr>
</tbody>
</table>

### 3.5 Protein Quantification

Cell lysates were collected using cell lysis buffer containing: 50 mM HEPES, 150 mM NaCl, 0.1M NaF (EMD Chemicals Inc., Darmstadt, Germany), 10 mM sodium pyrophosphate (Sigma-Aldrich), 0.25 M Sucrose (BioShop), 5 mM ethylenediaminetetraacetic acid (EDTA) (BioShop), 1 mM dithiothreitol (DTT) (BioShop), 1% protease inhibitor (Roche Diagnostics), 1% triton-X (BioRad), 1 mM Sodium orthovanadate (Sigma-Aldrich) and centrifuged at 16,000 x g for 10 minutes. Supernatant was collected and protein concentrations were determined using bicinchoninic acid (BCA) assay system (Pierce Biothechnology, Rockford, IL, USA). Protein quantification was performed as per manufacturers specifications.

### 3.6 Western Blot

Cell lysates were analyzed for UCP-1 (32 kDa) (Alpha Diagnostics Intl. Inc., San Antonio, Texas, USA), mitochondrial complexes I-V of the ETC, and β-actin (42 kDa)
(Cell Signaling, Danvers, MA, USA) protein by western blot. To probe for enzymes that make up the ETC (complexes I-V) an OXPHOS cocktail (Abcam, Cambridge, UK) was used which contained a mixture of antibodies that probed for all five enzymes (complexes I-V). 1μg of protein was separated using Mini-PROTEAN® Tetra Vertical Electrophoresis Cell (BioRad Laboratories, Hercules, CA, USA) on 10 or 12% (when probing for OXPHOS) SDS-PAGE gels. Proteins were then transferred to nitrocellulose (0.2μm) membranes (BioRad Laboratories) using a Criterion™ Blotter (BioRad Laboratories). Membranes were then placed in 5% BSA (BioShop, Burlington, ON, CA) in tris-buffered saline containing 1% Tween 20 (BioShop) (TBST) for 1 hour. Tris-buffered saline (pH 7.6) contained: 20mM Tris Base (Janssen Pharmaceuticals), and 150mM NaCl (Janssen Pharmaceuticals). After blocking, membranes were rinsed briefly in TBST and probed overnight with antibody dilutions in 5% BSA in TBST. Refer to Table 3 for antibody preparations. After overnight incubation, membranes were washed three times, 1-15-minute wash and 2-5-minute washes, with TBST and incubated for an hour with a horseradish peroxidase conjugated secondary antibody (Cell Signaling) diluted with 5% BSA in TBST. Following incubation with secondary antibody, membranes were washed with TBST, 1-15-minute wash, and 4-5-minute washes. Membranes were then incubated in SuperSignal West Femto substrate (Pierce Biotechnology) and chemiluminescent detection was used to visualize bands. ImageJ a free NIH imaging program (http://rsb.info.nih.gov/ij/index.html), was used to quantify bands of protein on membranes. β-actin was used as a loading control for UCP-1 and OXPHOS proteins. When probing for OXPHOS proteins, membranes needed to be
stripped and re-probed in order to acquire loading control (β-actin). To strip, membranes were incubated in Restore PLUS Western Blot stripping buffer (Life Technologies) for 30 minutes at 37°C. Following stripping, membranes were washed briefly in TBST and re-blocked for 1-hour before being placed in β-actin primary antibody overnight. Protocol is as previously described following incubation with primary antibody.

**Table 3: Antibody Preparations.**

<table>
<thead>
<tr>
<th>Antibody</th>
<th>Primary antibody dilution</th>
<th>Secondary antibody dilution (Anti-Rabbit/Mouse)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rabbit IgG UCP-1 (Alpha Diagnostics Intl. Inc., San Antonio, Texas, USA)</td>
<td>1:1000</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Mouse mAb OXPHOS (Abcam, Cambridge, UK)</td>
<td>1:5000</td>
<td>1:10,000</td>
</tr>
<tr>
<td>Rabbit mAb β-actin (Cell Signaling, Danvers, MA, USA)</td>
<td>1:5000</td>
<td>1:10,000</td>
</tr>
</tbody>
</table>

3.7 Whole-Cell Respiration Assay

TMCONs were differentiated in 75 cm² flasks and plated into 84 wells of a 96-well Flat bottomed OxoPlate (Precision Sensing GmbH, Regensburg, Germany) on day 8 of differentiation. Because mature adipocytes were unable to be counted efficiently, cells were plated at a density to ensure 100% confluency in the well on the day of the respiration assay. All work with OxoPlates was done in darkness, and all wells contained fresh media in order to hydrate sensors until day of experiment whether cells were present or not. On day 9 of differentiation, cells were treated with test compounds for either 24-hrs, or 6 days. After treatment, cells were washed briefly with PBS and 200μl/well of
oxygenated Minimal DMEM (Sigma-Aldrich) supplemented with: 10mM HEPES, 25mM glucose, 31mM NaCl, 2mM sodium pyruvate (Sigma-Aldrich), and 4% fatty-acid free BSA (100% respiration medium standard) was added to each well. Ten empty wells of the 96-well plate were used for calibration: 5-wells containing 100% respiration medium standard and 5-wells containing 0% O₂ Standard (10mg/ml sodium sulfite (Sigma-Aldrich) in ddH₂O). Cells were then placed in a SpectraMax M5 plate reader at 37°C for 45 minutes. After incubation, 75µl of mineral oil was laid over top of medium/calibration standards and fluorescence was read kinetically for 40 min (basal) using SpectraMax M5 plate reader. After basal oxygen levels were read, cells were injected with oligomycin (Sigma-Aldrich) (ATP synthase inhibitor) 2.5µM (final in well concentration), and fluorescence was read kinetically for 20 minutes. Injections were repeated to treat cells with final in well concentrations of 1µM carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP) (Sigma-Aldrich) a chemical uncoupler, and 2µM rotenone (Sigma-Aldrich) and 2µM antimycin A (Sigma-Aldrich) cocktail - complex I and III inhibitors respectively. These chemical modulators allow for ATP production respiration, uncoupled respiration, maximal respiration capacity and non-mitochondrial respiration to be measured. Oxygen consumption rates were determined as per manufacturer’s instructions.

3.8 Citrate Synthase (CS) Activity Assay

Brown adipocytes were differentiated in 12-well plates. Protein was quantified as outlined in section 3.4 and concentrations were used during final calculations to determine activity
of enzyme per μg of protein. Each sample was run in triplicate in a 96-well plate. Each reaction contained: 7.5μL of cell lysate, 12.5μL of cell lysis buffer (synthesized as described in section 3.4), 100μL of 0.2M Tris-HCl Buffer (pH 8.0) (Janssen Pharmaceuticals) supplemented with 0.2% (v/v) Triton-X 100 (BioRad), 20μL of 1mM 5,5’-Dithiobis-(2-nitrobenzoic acid) (DTNB) (Sigma-Aldrich), 2μL of 7.5mM acetyl-coA trilithium salt (Sigma-Aldrich), 48μL of ddH2O, and 10μL of 10mM oxaloacetic acid (Sigma-Aldrich). Tris-HCl supplemented with triton-x, DTNB, acetyl coA, and ddH2O solutions were combined and 170μL of reaction mixture was added carefully, to avoid bubbling, to each reaction containing sample. The 96-well plate was placed in SpectraMax M5 plate reader and absorbance was read at 412 nm wavelength for 3 minutes with 5 s of shaking before first read, and 3 s of shaking in between reads (background). Following background measurement, 10uL of 10mM oxaloacetate was added to each reaction mixture and absorbance was read by plate reader by the same parameters as background. The reaction was completed at 37°C and CS activity was calculated as follows:

$$ Activity = \frac{\Delta \text{Absorbance}_{After\ oxaloacetate} - \Delta \text{Absorbance}_{baseline}}{\text{extinction coefficient} \cdot [\text{Protein}] \cdot \text{volume of sample (μL)} \cdot \text{path length}} $$

An extinction coefficient of 0.0136 μmol⁻¹cm⁻¹ (DTNB), and a path length of 0.625cm, was used.
3.9 Cytochrome C Oxidase Activity Assay

TMCONs were differentiated in 12-well plates. Protein was quantified as outlined in section 3.4 and concentrations were used during final calculations to determine activity of enzyme per µg of protein. Each sample was run in triplicate in a 96-well plate. Each reaction contained: 12.5µL of cell lysate, 7.5µL of cell lysis buffer (as synthesized in section 3.4), 20µL of 0.1M potassium phosphate buffer (pH 7.0), 5µL of 40 mg/mL reduced cytochrome c, and 155µL of ddH₂O. Potassium phosphate buffer was synthesized by combining 0.1M potassium phosphate monobasic (Sigma-Aldrich), with 0.1M potassium phosphate dibasic (Sigma-Aldrich) (pH 7.0). Reduced cytochrome c was synthesized by adding 20µL of 10mg/ml sodium dithionate (Sigma-Aldrich) in potassium phosphate buffer solution to every 10mg of cytochrome c (Sigma-Aldrich) contained in a 40mg/ml solution of cytochrome c in potassium phosphate buffer. Potassium phosphate buffer, reduced cytochrome c, and ddH₂O was combined and 180µL of reaction mixture was added carefully, to avoid bubbling, to each reaction containing sample. Absorbance was immediately read at 550 nm wavelength for 3 minutes using SpectraMax M5 plate reader. Reaction was performed at 37°C and cytochrome c oxidase activity was calculated as follows:

\[
\text{Activity} = \frac{(\Delta \text{OD/min})}{(\text{protein (µg/µL)} \times \text{volume of sample (µL)})} \times \frac{\text{volume of reaction (L)}}{\text{extinction coefficient} \times \text{path length}}
\]

An extinction coefficient of 18.5 mmol⁻¹cm⁻¹ (reduced cytochrome c), a path length of 0.625cm, and a reaction volume of 0.0002 L was used.
3.10 Statistical Analysis

Statistical analysis and graphs were created using GraphPad Prism 6.0, (GraphPad Software Inc., San Diego, CA). Data were assessed for outliers using the Grubb’s test (GraphPad QuickCalcs, GraphPad Software Inc.). A 25% cut-off was chosen \textit{a priori} to identify pesticide hits in the three-tiered screening process (107). This parameter was consistent with percent-reduction seen by the control GW6471. Comparisons among two groups were analyzed using Student’s T-tests. Comparisons among multiple groups were analyzed using One-Way Analysis of Variance (ANOVA) followed by the appropriate post-hoc multiple comparisons test. Comparisons among multiple groups over multiple variables were analyzed using Two-Way ANOVA followed by the appropriate post-hoc multiple comparisons test. When equal variance failed, the Mann-Whitney Rank Sum Test or Kruskal-Wallis One-Way ANOVA on Ranks were used to determine significance. Alpha was set to equal 0.05 therefore significance was set at \( p \leq 0.05 \). All data are presented as mean \( \pm \) standard error of the mean (S.E.M).
CHAPTER 4: RESULTS

4.1 Pesticides and their primary metabolites reduce BAT UCP-1 promoter activity by greater than 25%.

To determine the direct effects of pesticides on BAT, a screen of 9 pesticides and 9 of their primary metabolites was performed using cells derived from UCP-1 luciferase reporter mice. Cells were treated with all compounds at either 1pM, 1nM, 1μM or three controls: vehicle (DMSO), 10μM isoproterenol, and 9μM GW6471 for 16 hours. A change (stimulation or inhibition) in UCP-1 promoter activity of greater than 25% versus vehicle control was considered a hit. At the 1pM concentration there were 4 compounds which inhibited UCP-1 promoter activity: chlorpyrifos, deltamethrin, glyphosate, and metam sodium (Figure 4a). After treatment at 1nM for 16 hrs, atrazine, chlorpyrifos, deltamethrin, glyphosate, imidacloprid (pesticides); chlorpyrifos oxon, and desethyl atrazine (pesticide metabolites) inhibited UCP-1 promoter activity (Figure 4b, Figure 5b). Four pesticides (chlorpyrifos, imidacloprid, metam sodium, permethrin) and six primary metabolites (atrazine mercapturate, 6-chloronicotinic acid, chlorpyrifos oxon, desethyl atrazine, MITC, 3-phenoxybenzoic acid) inhibited UCP-1 promoter activity after treatment for 16hrs at 1μM (Figure 4c, Figure 5c). None of the pesticides tested stimulated UCP-1 promoter activity. Results of all pesticides are summarized in Appendix Table 1. Compounds that reduced UCP-1 promoter activity at 1pM, and/or 1nM were further analyzed to determine their effects on Ucp-1 mRNA levels.
Figure 4. The effects of selected pesticides on UCP-1 promoter activity in BAT cells. Immortalized BAT cells derived from UCP-1 luciferase reporter mice were treated with pesticides at 1pM (a), 1nM (b), and 1μM (c) for 16hrs. UCP-1 promoter activity was determined by luciferase assay system (Promega). Controls for each experiment include vehicle (DMSO), 10μM isoproterenol (β-agonist), and 9μM GW6471 (PPARα antagonist). Data represent mean ± SEM (n=2, in duplicate)
Figure 5. The effects of selected pesticide metabolites on UCP-1 promoter activity in BAT cells. Immortalized BAT cells derived from UCP-1 luciferase reporter mice were treated with pesticide metabolites at 1pM (a), 1nM (b), and 1μM (c) for 16hrs. UCP-1 promoter activity was determined by luciferase assay system (Promega). Controls for each experiment include vehicle (DMSO), 10μM isoproterenol (β-agonist), and 9μM GW6471 (PPARα antagonist). Data represent mean ± SEM (n=2, in duplicate).
4.2 Pesticides and their metabolites reduced \( Ucp-1 \) mRNA levels in BAT cells derived from wild-type FVB/N mice

Six pesticides (atrazine, chlorpyrifos, deltamethrin, imidacloprid, metam sodium, glyphosate) and two of their primary metabolites (chlorpyrifos oxon, desethyl atrazine) entered a secondary screen to determine the direct effects of these compounds on \( Ucp-1 \) mRNA expression levels. A change (stimulation or inhibition) in \( Ucp-1 \) mRNA levels of greater than 25% versus vehicle control was considered a hit. BAT cells derived from wild-type FVB/N mice were treated with test compounds at 1pM, and 1nM for 4 hours. Atrazine, chlorpyrifos, deltamethrin and imidacloprid reduced \( Ucp-1 \) expression levels at 1pM and/or 1nM treatment (Figure 6a,b). Desethyl atrazine (pesticide metabolite) also reduced \( Ucp-1 \) expression levels by greater than 25% after 4-hours of treatment at 1pM (figure 6c) (refer to appendix table 2). These 5 compounds were further analyzed to explore their effects on UCP-1 protein expression levels.
Figure 6. The effects of pesticide and their metabolites on Ucp-1 mRNA expression in BAT cells. Immortalized BAT cells derived from FVB/N mice were treated with pesticides (a,b) and their primary metabolites (c,d), at 1pM (a,c), and 1nM (b,d) for 4 hrs to determine their effects on Ucp-1 mRNA levels. Relative mRNA levels were determined by RT-qPCR, corrected to Ppia. Controls include vehicle (DMSO), 10μM isoproterenol (β-agonist), and 9μM GW6471 (PPARα antagonist). Data represents the mean fold change from vehicle ± SEM (n=3, in triplicate).
4.3 Pesticides reduce BAT UCP-1 protein levels

Four pesticides were analyzed further in the tertiary screen where UCP-1 protein levels were analyzed using BAT cells derived from wild-type FVB/N mice. A change (stimulation or inhibition) in UCP-1 protein levels of greater than 25% versus vehicle control was considered a hit. Cells were treated with four compounds for 6 days at 1pM, and 1nM doses to assess UCP-1 protein levels. This time frame was established based on the observation that treatment of BAT cells with Gw, a PPARα antagonist, requires 6 days of treatment to observe a significant reduction in UCP-1 protein expression.

Atrazine, chlorpyrifos (CPF), and deltamethrin reduced UCP-1 protein levels at 1pM and/or 1nM (figure 7) (summarized in appendix table 3). These compounds also reduced UCP-1 promoter activity and mRNA expression (Figure 4, Figure 6, respectively). As CPF is a very commonly used pesticide that has been previously shown to cause metabolic disruptions such as weight gain and impaired insulin and leptin signaling (39, 65, 76, 79) this compound was selected as the main focus for the remainder of the thesis.
Figure 7. Effects of pesticides on UCP-1 protein expression in BAT cells.
Immortalized BAT cells derived from FVB/N mice were treated with pesticides (a,c) and desethyl atrazine (atrazine metabolite) (b,d) at 1pM (a,b), and 1nM (c,d) for 6-days. UCP-1 protein concentration was determined by western blot analysis. Data was corrected to β-actin. Controls include vehicle (DMSO), 10μM isoproterenol (β-agonist), and 9μM GW6471 (Ppara antagonist). Representative blots are on the right of each panel; data represented is mean ±SEM (n=3, in triplicate).
4.4 CPF significantly reduces BAT maximal respiration capacity after 6 days of treatment.

To further examine the effects of CPF on BAT function, whole cell respiration assays were completed following the treatment of BAT cells with CPF at 1pM, 1nM or 1μM for 24 hours (figure 8a), or 6 days (figure 8c). Oxygen consumption rates were assessed during successive injections with chemical modulators: refer to section 3.7 in methods. CPF significantly decreased maximal respiration capacity of BAT and UCP-1 protein expression following 6-day treatment (Figure 8c & d). To assess whether or not this reduction in BAT activity could also be observed with acute exposure, we determined respiration and UCP-1 protein levels after an acute (24 hour) exposure and compared this with the effects of chronic (6-day) exposure of CPF. Acute CPF exposure had no significant effect on either BAT maximal respiration capacity or UCP-1 protein levels (Figure 8a & b). These data suggest that chronic exposure to CPF is required to observe inhibition of BAT function. CPF did not significantly affect uncoupled respiration or non-mitochondrial respiration.
Figure 8. CPF significantly reduces BAT maximal respiration capacity after chronic treatment. Immortalized BAT cells derived from FVB/N mice were treated with vehicle, 1pM, 1nM or 1μM CPF for 24hrs (a), or 6 days (c) to assess its effects on mitochondrial respiration. Oxygen consumption rates (OCR) were analyzed during successive injections with chemical modulators (a,c). Data presented as fold change from basal OCR, mean ± SEM. OCR after injection with 1μM FCCP can be seen to the right of panels a and c. UCP-1 protein was analyzed in cells treated for 24hrs (b) or 6-days (d) normalized to β-actin. *P<0.05 (n=3) relative to vehicle calculated by Two Way ANOVA followed by Dunnett’s multiple comparisons test.
4.5 CPF metabolites significantly reduce BAT maximal respiration capacity after 24 hrs.

In order to assess direct effects of CPF metabolites on BAT mitochondrial respiration a whole cell respiration assay was performed. Brown adipocytes were treated with CPF metabolites: CPF oxon (figure 9a) and TCP-y (figure 9b), at 1pM, 1nM or 1μM for 24 hours. Oxygen consumption rates were assessed during successive injections with chemical modulators (oligomycin, FCCP, and rotenone/antimycin A). Injection with FCCP (a chemical uncoupler) demonstrates that CPF metabolites significantly reduce maximal respiration capacity of cells after 24 hours of treatment. CPF metabolites did not significantly affect ATP production/uncoupled respiration or non-mitochondrial respiration.
Figure 9. CPF metabolites significantly reduce maximal respiration capacity after acute treatment. Immortalized BAT cells derived from FVB/N mice treated with vehicle, 1pM, 1nM or 1μM of CPF oxon (a) or TCP-y (b) for 24hrs. Oxygen Consumption Rates (OCR) of cells were analyzed during successive injections with chemical modulators to assess mitochondrial respiration. OCR following 1μM FCCP injection can be seen at the right of each panel demonstrating reduction in total mitochondrial respiration capacity. Data presented as fold change from basal OCR, mean ± SEM. *P<0.05 (n=3) relative to vehicle calculated by Two Way ANOVA followed by Dunnett’s multiple comparisons test.
4.6 CPF significantly increases stimulated UCP-1 protein expression in BAT

In order to assess the effects of CPF on basal and stimulated UCP-1 mRNA and protein expression, cells were co-treated with 1pM or 1nM CPF and Iso for 4.5-hrs (mRNA analysis) or 6 days (protein analysis). 1pM CPF was unable to significantly alter stimulated UCP-1 mRNA or protein expression levels (Figure 10 a, b). 6-day co-treatment with 1nM CPF and Iso significantly increased stimulated UCP-1 protein expression levels (Figure 10d). This suggests that the inhibitory effects of CPF on BAT function are independent of the β-adrenergic/cAMP signaling pathway that primarily activates BAT.
Figure 10. Effects of CPF in combination with isoproterenol on UCP-1 protein expression. Immortalized BAT cells derived from FVB/N mice were co-treated with 1pM (a,c) or 1nM (b,d) CPF, and 0.1, 1, or 10µM isoproterenol for 4.5 hrs (a,b) or 6 days (c,d). Adipocytes treated for 4.5hrs. were analyzed for Ucp-1 levels. Adipocytes treated for 6 days were analyzed for UCP-1 protein. Relative mRNA levels were determined by RT-qPCR and corrected to Ppia. Representative blots are on the right of panels c and d and corrected to β-actin. Data represents the mean± SEM (n=3).
4.7 CPF significantly reduces mRNA expression of genes important for mitochondrial oxidative phosphorylation and biogenesis

In order to explore the mechanism by which CPF reduces mitochondrial function, cells were treated with CPF (1pM and 1nM) or vehicle for 4-hrs to determine the mRNA levels of Cox8b, Cox2, Cs, Ppargc1α and Hadh - genes important for mitochondrial oxidative phosphorylation and biogenesis. 1pM CPF significantly decreased Cox8b, Cox2, and Ppargc1α expression levels (Figure 11a). Also, 4-hour treatment with 1nM CPF significantly decreased Ppargc1α expression levels (Figure 11b). CPF did not significantly change expression of Cs or Hadh, genes upstream of the ETC.

Figure 11. CPF significantly reduces mRNA expression of genes important to mitochondrial oxidative phosphorylation and biogenesis. Immortalized BAT cells derived from FVB/N mice were treated with Vehicle (DMSO), 1pM (a), or 1nM (b) CPF for 4 hrs. 4-hrs treated adipocytes were analyzed to determine relative mRNA levels of Cox8b, Cox2, Cs, Ppargc1α, and Hadh by RT-qPCR, corrected to Ppia. Data represents the mean ± SEM. *P<0.05 (n=3) relative to vehicle calculated by Student’s T-test.
4.8 CPF significantly increases Complex III and IV protein expression.

In order to assess the direct effects of CPF on proteins that make up the ETC, cells were treated with 1pM, 1nM CPF or vehicle for 6-days and analyzed by western blot. There was no significant effect of CPF at either dose tested on the expression of CI, CII, or CV. Treatment with 1nM CPF but not 1pM CPF significantly increased the expression of complex III and complex IV proteins.

Figure 12. CPF increases the expression of mitochondrial ETC proteins.
Immortalized BAT cells derived from FVB/N mice were treated with Vehicle (DMSO), 1pM (a), or 1nM (b) CPF for 6-days. 6-day treated adipocytes were analyzed to determine protein levels of enzymes that make up the ETC of the mitochondria (Complexes I-V) by western blotting corrected to β-actin. Representative blots can be found in panel c. Data represents the mean ± SEM. *P<0.05 (n=3) relative to vehicle calculated by Student’s T-test.
4.9 CPF significantly reduces cytochrome c oxidase activity.

In order to determine the effects of CPF on cytochrome c oxidase, and citrate synthase activity, cells were treated with 1pM, or 1nM CPF for 6-days. Analysis of citrate synthase activity allows disruptions in mitochondrial substrate availability and changes in mitochondrial number to be detected. Investigating cytochrome c oxidase activity (complex IV) allows disruptions in electron transfer and oxidative phosphorylation to be detected. These measures show functional effects of CPF on enzymes important to mitochondrial function. 1pM CPF significantly decreased cytochrome c oxidase activity. CPF did not alter citrate synthase activity at either dose tested (Figure 12a).

![Graphs showing the effects of CPF on citrate synthase and cytochrome c oxidase activities.](image)

**Figure 13. CPF significantly reduces Cytochrome C Oxidase activity.** Immortalized BAT cells derived from FVB/N mice were treated with Vehicle (DMSO), 1pM (a), or 1nM (b) CPF for 6 days. Enzymatic activity was determined by assay systems specific to citrate synthase (a) and cytochrome c oxidase (b) and normalized by total protein content. Total protein content was determined by BCA assay. Data represent mean ± SEM. *P<0.05 (n=3) relative to vehicle calculated by One Way ANOVA followed by Dunnett’s multiple comparison’s test.
CHAPTER 5: DISCUSSION

5.1 The effects of pesticides on mature BAT UCP-1 expression levels

The off-target toxicity of pesticides on human health is a rapidly growing area of investigation. Although there are contradicting studies on the effects of these compounds on metabolic function one cannot ignore the growing body of work linking pesticides and metabolic diseases, such as obesity and type-2 diabetes (35, 38, 48, 52, 58, 59, 62 - 64, 80 - 86). Cell culture, animal and human epidemiological studies continue to present the link between pesticides and metabolic perturbations. However, few of these works have investigated the low dose effects of these compounds especially in the context of their effects on BAT. Scientists have demonstrated the importance of BAT thermogenesis in energy homeostasis and its potential as a target for therapies targeting obesity and ameliorating glycemic control (5, 8, 11, 13 - 15, 18, 20 - 24, 27, 29, 87). The results of my work demonstrate that treatment of BAT cells in vitro with commonly used pesticides at levels which are relevant to human exposure (i.e., general and occupational exposure) can significantly inhibit BAT activity (39). The three-tiered screening process of nine pesticides and nine of their primary metabolites revealed that atrazine, deltamethrin and chlorpyrifos consistently reduced UCP-1 expression levels at 1pM and/or 1nM. This, to my knowledge, is the first report of the effects of pesticides on UCP-1 expression in BAT, a key protein for BAT thermogenesis.
Atrazine is a triazine herbicide known to act via the inhibition of photosystem II in the thylakoid membrane of chloroplasts, a structure very similar to the mitochondria (34, 68). Mitochondria play a central role in BAT thermogenesis and therefore perturbations of this organelle, may result in reduced expression of proteins important for BAT function or loss of function of this tissue as a whole. My work demonstrates that atrazine has the potential to affect BAT function and this affect may be due to reduced UCP-1 expression and mitochondrial uncoupling. Several studies have shown the link between atrazine and mitochondrial dysfunction. Indeed Lim, S et al., showed in rats that chronic exposure to atrazine resulted in decreased basal metabolic rate, increased body weight, increased intra-abdominal fat and elevated insulin resistance of normal-fed rats exposed over 5 months (59). They also showed that the atrazine-induced insulin resistance and obesity was exacerbated when rats were on a high-fat diet. Similarly, epidemiological studies show an overlap in areas with high usage of atrazine and the prevalence of individuals with a BMI of over 30kg/m² (34, 68). Taken together these data suggest that atrazine exposure may contribute to obesity and aberrant glycemic control. Results from my study suggest that inhibition of BAT activity by atrazine may play a role in these observed metabolic perturbations (particularly the reduced basal metabolic rate). However, the contribution of reduced BAT function to atrazine-mediated obesity in animal and epidemiological studies has yet to be examined.

Deltamethrin is a type-II pyrethroid insecticide, which binds to voltage sensitive sodium channels in neurons causing prolonged activation and resulting in convulsion, paralysis
and death of target organisms (86). This compound is of interest as it is thought of as a “safer” alternative to carbamate and organophosphorus insecticides and is therefore used extensively around the world (86). My present work suggests that deltamethrin can potentially reduce BAT activity by reducing uncoupled respiration via a reduction in UCP-1 expression. In a study by Shen et al., deltamethrin significantly increased lipid accumulation, decreased the activity of the AMP-activated kinase (AMPKα) and the inhibitory phosphorylation of acetyl-CoA carboxylase (ACC), while increasing the expression of CCAAT/enhancer-binding protein (C/EBPα) and PPARγ in 3T3-L1 adipocytes (86). In another study, deltamethrin induced endoplasmic reticulum (ER) stress via increased intracellular calcium levels in SK-N-AS neuroblastoma cells (38). Interestingly, calcium influx is known to alter the adrenergic signaling pathway important to BAT thermogenesis and UCP-1 expression, while also being a known upstream regulator of AMPKα (86, 88). This demonstrates that deltamethrin may have the capability of altering lipid metabolism via changes in calcium influx that could potentially alter AMPKα and adrenergic signalling. AMPKα is the master regulator of energy metabolism and has been shown to be involved in BAT development and function (19). Taken together with the results in my study, deltamethrin may be altering calcium release in BAT reducing UCP-1 expression in a manner that may be mediated by AMPKα, potentially causing reduced BAT activity that may translate into metabolic disturbances.
5.2 CPF

CPF is a lipophilic, organophosphorus insecticide used to control pests (e.g. cockroaches, fleas, termites, mosquitos) in agriculture; reduce insect damage on turf, lawns and golf courses; and is sometimes a component in tick and flea collars for pets (39). CPF is mainly used in agriculture for corn, tree nuts, and soy bean crops (39). This compounds first came to the market in 1965 and rapidly became one of the most widely used insecticides, with the U.S making up 60% of total worldwide use (39). CPF’s primary metabolite (i.e., CPF oxon) irreversibly binds to and inhibits acetylcholinesterase (AChE) (39, 89,90). AChE catabolizes acetylcholine (a neurotransmitter) and therefore terminates synaptic function in neuronal tissues (39,89). Although the main function of this pesticide is to inhibit AChE, CPF has been shown to inhibit several other esterases (e.g. butylcholinesterase, carboxyesterases, serine hydrolase), alter adenylyl cyclase signaling, cause genotoxicity (via direct DNA damage), generate ROS, and cause lipid peroxidation; all mechanisms that have been previously shown to contribute to the development of chronic diseases such as obesity and type-2 diabetes (39, 63, 79, 90 - 92). Characteristics of a CPF overdose include sweating, salivation, bronchoconstriction, increased bronchial secretion, elevated gastrointestinal motility, diarrhea, muscular twitching, and tremors along with several other nervous system effects (39). All signs that must be monitored when studying CPF in vivo (39).
CPF is oxidized into its major metabolite CPF oxon, and then is either spontaneously or enzymatically hydrolyzed into its secondary metabolites 3,5,6–trichloro-2-pyridinol (TCPy) and diethylphosphate (39). CPF can also be oxidized by cytochrome P-450 into an unstable intermediate that is rapidly hydrolyzed into diethylthiophosphate and TCP-y (39). Its secondary metabolites are then excreted in the urine (39). Pharmacokinetic studies reveal that ~70% of CPF is absorbed in the body following oral administration, with most of the compound distributing to fat tissues (39). Toxicologists have not been able to accurately quantify the half-life of CPF in adipose tissue. Other tissues in which an appreciable amount of CPF can accumulate are brain, liver and kidney (high to low) (39). Although CPF quickly volatizes in the atmosphere, CPF is lipophilic and quickly binds to soil or plant particles (39). When in the environment, CPF can persist for up to 3 days, while indoors, CPF can persist for several months, this is due to lack of sunlight, water, and/or soil microorganisms which rapidly degrade the compound (39). Given its high usage in commerce there is high potential for widespread exposure to CPF (39).
Like most pesticides, humans are exposed to CPF via dermal, inhalation, and ingestion pathways with ingestion being the most common non-occupational exposure pathway (39). In 2000, the U.S. Environmental Protection Agency (EPA), established the reference dose (maximal acceptable oral dose of a toxic substance) of 0.3µg/kg of body weight/day for CPF (39). While there is an established reference dose for CPF it is very difficult to determine the precise quantity of CPF that individuals today are being exposed to.

Previous studies have used urinary TCPy (a metabolite of CPF) levels as a measure of CPF exposure (39). However, TCPy is a major metabolite of several other compounds (39). A study in 2016 estimated that occupational exposure in agricultural workers to CPF was estimated to be 3.70µg/kg of bodyweight/day which is higher than the recommended guidelines (92). Although occupational exposure may be high, most recent exposure studies estimate that typical daily adult exposure ranges from 0.004-0.006µg/kg of body weight/day with the majority distributing to fat tissue but also the brain (39). Human studies with exposure levels of 3µg/kg of body weight/day (similar to occupational exposure levels), estimated there was 0.01nM of CPF in blood, and 0.3nM in the brain (39). My study investigated the effects of CPF on BAT at 1pM and 1nM doses. One can infer based on these mentioned pharmacokinetic and distribution studies, that the doses that have been tested in my study (i.e., 1pM and 1nM) may be representative to what is found in fat of those exposed in the general public (1pM) and occupationally (agricultural) exposed individuals (1nM) (39). Although, further studies using different markers of CPF exposure must be performed to be certain.
In my study, CPF inhibited maximal respiration capacity of BAT (measure of BAT function) at all doses tested (1pM, 1nM, and 1μM); including doses which are relevant for human exposure levels. This effect appeared to be dependent on a longer exposure period (i.e., 6-days) and accompanied by a reduction in basal UCP-1 expression. If similar effects are seen in vivo, these data suggest that CPF exposure has the potential to reduce BAT activity and potentially contribute to obesity and its associated co-morbidities. Indeed, there have been animal studies which support this hypothesis; CPF exposure in rodents has been shown to cause increased body weight and aberrant glucose control (76, 82, 83, 93 - 95). For this reason, CPF became the main focus of my study following the identification of pesticides that have the potential to alter BAT function.

There are very few epidemiological studies investigating the associations between CPF and metabolic disease. One of the most informative epidemiological studies on chlorpyrifos was that of Lee, et al., who investigated all-cause mortality in pesticide applicators exposed to CPF (82). In this study, they reported that although not statistically significant, any CPF exposure was associated with increased risk of death from metabolic diseases with a relative risk reported as 1.45 (95% CI, 0.80–2.63) (82). The bulk of research on the association of CPF and metabolic perturbations is focused on the in vivo and in vitro effects on neuronal, heart and liver tissues. Although there are several studies looking at the effects of CPF on these tissues, few are in the context of metabolism.
Studies that looked at the effects of CPF on metabolism include: a study by Peris-Sampedro, F. et al (76). In this study they exposed wild-type and apoE3 mice to 2mg/kg of body weight/day of CPF for 8 weeks in order to gain further insight on the effects of CPF on metabolism. They determined that CPF-treated apoE3 mice exhibited increased weight gain, food intake, total cholesterol, fasting glucose and insulin resistance. This investigation revealed that not only does CPF alter glucose and lipid metabolism in exposed groups, it also alters feeding behaviors in adult male mice treated at doses devoid of cholinergic toxicity. Effects on energy expenditure were not investigated. Similarly, two studies assessing the effects of high-fat diet fed mice exposed to 2.0mg/kg CPF acutely (single dose) or chronically (10 d.) found that CPF inhibited carboxylesterase, and fatty acid amide hydrolase activity (non-cholinergic endpoints) and reduced expression of genes important in de novo lipogenesis and cytochrome P450 enzymes in mouse liver (83, 93). Inhibition of carboxylesterase and fatty acid amide hydrolase was exacerbated in high fat diet fed mice and these effects were confirmed in vitro using primary rat hepatocytes exposed to 20μM CPF (83). My study provides another pathway by which CPF could influence metabolic outcomes and are consistent with an adverse (obesogenic) impact of this chemical on body weight homeostasis. Through my three-tiered screening process I have identified CPF as a potential inhibitor of BAT activity. Negative regulators of BAT (e.g., Liver X receptor α; LXRα, Vitamin D receptor; VDR, receptor interacting protein 140; RIP 140) have been previously shown to cause reduced energy expenditure, decreased UCP-1 expression and increased susceptibility to diet-induced obesity in mice (94). The effects of CPF on whole-body metabolism still remain unclear as studies show
contradictory results at high exposure levels (above occupational exposure levels). This can be attributed to differences in exposure route, dose, and species being studied. More investigations are required to determine the direct effects of CPF on metabolism, including studies using low exposure levels that are more relevant to human daily (0.004-0.006 μg/kg-day) or occupational (~3.7 μg/kg-day) exposure levels (39). This is important as environmental toxicants have often followed non-monotonic dose response relationships, where they may show more potent effects at much lower exposure levels (95). My study highlights the importance of evaluating biologically relevant dose effects when investigating the toxicology of environmental toxicants as it shows that CPF is altering BAT function at levels seen in occupational settings in agriculture (39).

5.3 The effects of CPF on β-adrenergic signaling pathway in mature BAT

In order to determine the mechanism by which CPF imparts its effects on BAT it was important to determine if this compound affects the β-adrenergic/cAMP signaling pathway that plays a central role in BAT thermogenesis. BAT tissue activation occurs via β-adrenergic receptors (G-proteins) that act through AC (8). CPF has been previously shown to affect the AC signaling cascade in brain and heart tissue by altering AC expression and activity, and the function of G-proteins that link hormone receptors to AC (79). The reduction in UCP-1 expression levels seen throughout my 3-tiered screening process demonstrates a possibility that CPF may alter BAT activity via the β-adrenergic/cAMP signaling pathway in which AC is a major player. In order to determine the effects of CPF on this signaling cascade, BAT cells were co-treated with CPF and
isoproterenol. Isoproterenol (Iso) is a β-agonist that stimulates the β-adrenergic/cAMP signaling pathway resulting in a dose-dependent increase (that maxes out at 1μM) in UCP-1 expression, lipolysis, and expression of several other genes that are important to thermogenesis (8). 1pM and 1nM CPF treatment present different effects when co-treated with isoproterenol depending on dose. Although CPF significantly decreased basal UCP-1 expression levels, 1pM CPF had no significant effect on Iso-stimulated UCP-1 expression. This suggests that at 1pM, CPF is not affecting the β-adrenergic/cAMP signaling pathway. Although contrary to screening data, the effects of 1pM CPF on stimulated UCP-1 expression is in accordance with respiration data. Post oligomycin injection (ATPase inhibitor), and rotenone/antimycin A cocktail (complex I and III inhibitors) injection, we see no changes in the respiration between control and treated cells. This suggests that there are no changes in uncoupled respiration or ATP production after treatment with 1pM CPF. Interestingly, 1nM CPF stimulated UCP-1 expression even further when co-treated with isoproterenol, thus indicating that CPF may be affecting a down-stream signaling cascade and further stimulating adrenergic induced UCP-1 expression. This is contrary to data from our screen where we see CPF decreased basal UCP-1 expression suggesting, that CPF’s inhibitory effect on UCP-1 expression occurs in a manner that is independent of β-adrenergic/cAMP signaling. This is also contrary to respiration data from 1nM CPF treated BAT, in which no changes in respiration were seen post oligomycin and rotenone/antimycin A cocktail injection. This suggests that there are no changes in uncoupled or ATP respiration following 1nM CPF treatment. These data show that although there are effects of 1nM CPF on stimulated
UCP-1 expression, these effects do not translate to a functional outcome. These data are contrary to the study performed by Song, X., et al in 1997 as their data showed decreases in AC signaling following CPF exposure (79). The differences between data from Song, X., et al.'s and my study may be attributed to higher exposure levels (higher than reference dose and occupational exposure levels) and the tissues being analyzed in their study (i.e. brain and heart tissue) (79). The increased expression of UCP-1 after co-treatment with 1nM CPF and isoproterenol, could be attributed to a compensatory effect where the adipocytes are trying to stimulate respiration by increasing ETC complex expression or mitochondrial number as a result of CPF-induced decreases in maximal respiration capacity (figure 8c). Compensatory action by mitochondria (i.e. proliferation of mitochondria) to stimulate respiration often occurs when mitochondria are not functioning properly (72). My data demonstrated that the effects of CPF on BAT, i.e. decreased in UCP-1 expression are not as a result of changes in the β-adrenergic/cAMP signaling pathway and that the reduction in maximal respiration capacity was not dependent on the downregulation of UCP-1. Downregulation of maximal respiration capacity is an indicator of mitochondrial dysfunction. Therefore, analyzing the effects of this compound on mitochondrial number, mitochondrial ETC complex expression, activity, and bioenergetic substrate transport are important to determining how CPF is affecting BAT.
5.4 The effects of 1pM CPF on mature BAT mitochondrial function

It has been previously suggested that CPF can alter mitochondrial function in several other tissue types such as the heart and brain. In a study by Lee et al., CPF at varying doses (0-200µM) was shown to inhibit complex I and cause mitochondrial mediated apoptosis as a result of ROS production in PC12 cells (neuronal in vitro model) (65). A study where cortical neurons of CPF and CPF oxon exposed rats were analyzed, demonstrated that CPF and its metabolite were able to dose-dependently decrease membrane potential, decrease mitochondrial number and increase mitochondrial length (64). In addition to decreases in UCP-1 expression, my study demonstrated that CPF reduces maximal respiration capacity in BAT exposed for 6 days. FCCP is a chemical uncoupler that disrupts mitochondrial membrane potential resulting in uninhibited electron flow through the ETC via complex IV (32). Injection of this compound while assessing oxygen consumption rates of CPF exposed BAT allows maximal respiration capacity of cells to be determined. The reduction in BAT maximal respiration capacity of 6-day CPF exposed cells indicates that CPF is altering mitochondrial function. Figure 11a demonstrates that 1pM CPF is able to significantly reduce genes important for mitochondrial oxidative phosphorylation, and biogenesis, but not genes important to the TCA cycle or beta oxidation which are important for production of reducing equivalents (i.e. NADH and FADH$_2$) for the ETC. 1pM CPF significantly reduced genes important for nuclear and mitochondrial encoded subunits of complex IV. Furthermore, CPF significantly reduced Ppargc1α which encodes for PGC1α. PGC1α is a master transcriptional regulator of mitochondrial metabolism, specifically mitochondrial
biogenesis, cellular respiration rates, and energy substrate uptake and utilization (71,96).

PGC1α coactivates nuclear respiratory factor-1 (NRF-1) and -2 (NRF-2) which in turn regulates the transcription of mitochondrial transcription factor A (Tfam) and other nuclear encoded genes important in mitochondrial function (such as UCP-1) (8,94,96). The observed difference in PGC1α could explain why CPF exposure caused a decrease in UCP-1 at both 1pM, and 1nM, as the reduction in Ppargc1α can be seen after exposure to either dose. Studies in PGC1α knock-out mice demonstrated that this protein is essential for BAT activation/thermogenesis (94). Gene expression data suggests that 1pM CPF may be reducing maximal mitochondrial respiration capacity via changes in mitochondrial number or mitochondrial function of proteins that make up the ETC, specifically complex IV.

Mitochondrial dysfunction can occur as a result of diminished mitochondrial content; diminished substrate availability; decrease in mitochondrial activity as a result of inhibition of proteins that make up the ETC; changes in mitochondrial morphology; or finally, because of damage to mitochondria as a result of ROS generation (71). No significant changes in Hadh (gene important for β-oxidation) and Cs (gene important to the TCA cycle) genes after treatment with 1pM CPF suggests that CPF is not affecting substrate availability. In order to determine if CPF is downregulating the expression of ETC proteins, the expression of proteins that make up the ETC were analyzed. 1pM CPF had no effects on any of the proteins that make up the ETC, this includes complex IV which gene expression was previously noted to be significantly reduced. This suggests
that 1pM CPF is not altering the expression of proteins that make up the ETC. This is contrary to other reports which have shown decreases in expression levels of ETC proteins upon exposure to other organophosphorus insecticides (64). Contradicting data could be attributed to the differences between the studies such as the tissue being analyzed (i.e. brain, heart, liver), doses and compounds tested (64). Figure 13b shows that 6-day treatment with 1pM CPF decreases complex IV (cytochrome c oxidase) activity in mature BAT. Similar results have been shown in a study by Basha and Poojary, 2014 (69). They demonstrated that sublethal doses of CPF significantly inhibited complex IV enzyme activity in the brain of rats of varying age groups (69). Furthermore, their study showed that the effects of CPF were further exacerbated upon cold exposure. Although, these effects were seen at doses higher than those estimated by occupational exposure.

Other organophosphorus insecticides have also been shown to alter the activity of enzymes that make up the ETC. For example, mevinphos treatment caused a dose-dependent decrease in the activity of complexes I-IV in neuronal cells and monocrotophos and dichlorvos inhibited complex I, II and IV activities in the cortex, cerebellum, and brain stem tissues (64). Taken together, my data shows that the reduction in maximal respiration capacity after treatment with 1pM CPF may be as a result of decreases in complex IV activity.

Cytochrome c oxidase (complex IV) is an extremely complex enzyme, made up of 13 subunits (97, 98). Subunits I-III are encoded by mitochondrial DNA, synthesized in the mitochondria, and form the catalytic core, while the remaining subunits are nuclear
encoded and are synthesized in the cytoplasm (97, 98). The mechanism of assembly of this enzyme is still under extensive investigation, although it is known that it requires the assistance of greater than 20 additional nuclear encoded factors that act at all levels of the assembly process (97 - 99). Consequently, there are several mechanisms by which CPF may be inhibiting complex IV activity. Such means may include: changes in expression of factors important for complex IV assembly, mitochondrial morphology that affect the assembly process, or damage of the enzyme via the production of ROS. It has been previously suggested that CPF can alter mitochondrial length in a dose-dependent manner in cortical neurons (64). Furthermore, other organophosphorus insecticides such as diazinon and dichlorvos have been shown to alter the cristae of mitochondria in liver and CNS of exposed rats (64). Additionally, ROS can damage mitochondrial DNA, cause protein aggregation, and lipid peroxidation which could lead to the removal of damaged mitochondria (mitophagy), or under high stress conditions apoptosis (65, 71). CPF and several other organophosphorus insecticides are known to increase ROS production, and it is even believed that induction of oxidative stress is an important mechanism by which these insecticides exert their action (64 - 67). A study by Elsharkawy, E, et al., demonstrated a significant increase in ROS production in the liver of rats exposed to a single dose of CPF, as a result of significant decreases in total antioxidant levels (67). Several organophosphorus insecticides have been shown to induce oxidative stress in rats and humans, specifically, lipid peroxidation has been evident in rat brains and erythrocytes, that have led to seizures and tubular necrosis (66). Furthermore, organophosphorus insecticides have also been shown to disturb antioxidant systems,
mechanisms put in place to reduce ROS production (64). These studies suggest that CPF may be inhibiting complex IV activity via increased oxidative stress or changes in mitochondrial morphology although this remains to be determined.

5.5 The effects of 1nM CPF on mature BAT mitochondrial function

There is an increasing awareness that consideration of both dose and duration of exposure are important factors when studying endocrine disruptors such as pesticides. My work further highlights the importance of dose and duration of CPF exposure on BAT function. 1nM CPF is the most effective dose at reducing maximal respiration capacity of mature BAT cells. Interestingly, it is at this dose where no changes in the expression of genes important to mitochondrial function or mitochondrial complex IV activity are seen. This is in contrast to the effects seen after treatment with 1pM CPF where we see a drop in the expression of genes that encode for enzymes of the ETC and \textit{Ppargc1a}, along with a reduction in complex IV activity. This suggests that the mechanism of action of 1nM CPF is different from the mechanism of action of 1pM CPF. BAT cells treated with 1nM or 1pM CPF demonstrate how the reduction in maximal respiration capacity is not attributed to a reduction in the expression of proteins that make up the ETC. On the contrary, a significant increase in the expression of complex III and IV is seen when exposed to 1nM CPF. This may be attributable, in part, to compensation for the reduction in respiration capacity. Several reports have shown that significant mitochondrial dysfunction is at times accompanied by compensatory mitochondrial proliferation, or increased expression of proteins that make up the ETC (72). Effects such as these have been exhibited in
human diseases attributed to mitochondrial DNA mutations, where there is an accumulation of abnormal mitochondria in the fibers of skeletal muscle (100).

Furthermore, figure 13 has also shown that 1nM CPF had no effect on the activity of enzymes important to the TCA cycle or components of the ETC. This further emphasizes that at this dose CPF is not affecting structural components essential for mitochondrial function, but rather may be affecting the electrochemical gradient that drives oxidative phosphorylation. Although, further studies must be performed to make this conclusion.

Organophosphorus insecticides, including CPF and its metabolite CPF oxon have been shown to decrease mitochondrial membrane potential in neuronal tissues (64). Since mitochondrial membrane potential influences/impacts maximal mitochondrial respiration capacity it is possible that the reduction in maximal respiration capacity after treatment with 1nM CPF is as a result of changes in membrane potential of the mitochondria.

However, this remains to be tested.

5.6 The effects of CPF metabolites on mature BAT mitochondrial function

An interesting finding of my study was the differences in effect that were seen between CPF and CPF metabolites on BAT activity. CPF required a long-term exposure of 6-days to impart its effects on BAT activity and UCP-1 expression. By contrast, acute exposure (i.e., 24-hrs) with CPF metabolites: CPF oxon and TCP-y, were sufficient to significantly decrease maximal respiration capacity of these cells but were unable to reduce basal UCP-1 expression levels. Additional analysis is required to further understand what is occurring in the BAT cells when treated with CPF metabolites. Although, this is not the
first account in which CPF metabolites show more toxic effects than its parent. Indeed, the main mode of action of CPF is via its primary metabolite CPF oxon (39). CPF oxon, not its parent CPF, binds irreversibly to AChE to inhibit acetylcholine turnover, leading to death of pests in agriculture (39). Interestingly, TCP-y is a metabolite for several insecticides, not just CPF, therefore adverse effects by this compound is of concern as it is a by-product of many pesticides and many tissues may be exposed to this compound more frequently (39). Furthermore, studies on the effects of TCP-y on mitochondrial function, to my knowledge, have not been reported. Similar to CPF, its major metabolite CPF oxon has also been shown to cause mitochondrial dysfunction (64). It is important in future studies to determine whether the effects of CPF oxon on mitochondrial function is a result of changes in mitochondrial number, ETC enzyme activity, or changes in membrane potential. Furthermore, the adverse effects seen by both parent and metabolites demonstrate that if these results can be replicated in vivo, CPF-induced BAT inhibition may cause impaired lipid and glucose homeostasis that may lead to metabolic diseases such as obesity and type-2 diabetes.

5.7 Implications

My work is, to my knowledge, the first account of the effects of pesticides of varying classes (i.e. insecticides, herbicides, fungicides) on BAT function. Between the years of 2002 and 2006 approximately 4 million kg of CPF was used, almost exclusively for agricultural purposes (39). We are readily exposed to CPF primarily via residues on food we consume, but also via contamination of the environment such as ground water, soil, or
the air post agricultural applications (levels which vary from community to community) (39). Its hydrophobicity makes it more sensitive to the elements and also allows this compound to persist on contaminated surfaces in homes for long periods of time (39). CPF reduces UCP-1 expression levels, a protein central to adaptive thermogenesis in BAT, at doses of biological relevance. Estimates show that these levels may be seen in individuals who are occupationally exposed (8, 39, 94). Furthermore, CPF and its primary metabolites (CPF oxon, TCPy) reduce maximal respiration capacity (BAT function) of BAT in mechanisms that vary based on dose and duration of exposure, suggesting that CPF may be causing mitochondrial dysfunction in exposed adipocytes via multiple mechanisms.

BAT activation is now being studied as a new method of combating the obesity epidemic, as studies have now shown that active BAT can consume up to 20% of basal caloric needs (94). This being said BAT deactivation may lead to metabolic diseases such as obesity. Indeed, studies have now shown a correlation between decreased BAT mass and activity and obese or aged subjects (14, 94). In mice, genetic deletion of negative regulators of BAT such as RIP 140, LXRα, TPH1 and VDR results in a lean phenotype, resistance to diet-induced obesity and BAT containing higher levels of UCP-1 (94). Overstimulation of VDR in BAT was characterized by reduced energy expenditure, suppression of genes involved in thermogenesis and fatty acid oxidation in both WAT and BAT, all characteristics that lead to an obese phenotype (94). My work suggests that exposure to CPF has the potential to reduce BAT activity and therefore may decrease
energy expenditure. This may lead to or augment an obese phenotype, similarly to what is seen when BAT negative regulators are activated.

In addition to decreases in UCP-1 expression levels, CPF exposure also decreased maximal respiration capacity, complex IV activity and altered complex protein expression. All changes which have been previously shown by other insecticides of its class in other tissues including the heart, neurons, and liver (64). Many studies have suggested that mitochondrial dysfunction is central to the pathogenesis of insulin resistance and metabolic syndrome (68). Mitochondrial abnormalities, such as altered mitochondrial gene expression, and oxidative phosphorylation have been seen in skeletal muscle, liver and fat tissues of obese and insulin resistant individuals (68, 71). Additionally, reduced oxidation of fuels as a result of mitochondrial dysfunction has been associated with intracellular lipid accumulation that can alter insulin signaling which can contribute to obesity and type-2 diabetes (68, 71). Diacylglycerol, and ceramides (metabolically active lipid mediators), inhibit insulin signaling via protein kinase C (PKC)-mediated inhibition of the insulin receptor and PKB, respectively (71). Furthermore, damage to mitochondrial components as a result of factors such as ROS, can result in mitophagy, reduction in mitochondrial density and apoptosis, which has also been associated with insulin resistance (68, 71). Taken together my work has identified a novel BAT inhibitor (i.e., CPF) which can potentially contribute to or produce an obese/diabetic phenotype. Several other insecticides of its class have also been shown to carry out similar effects on mitochondria in other tissues (i.e., brain, heart, liver) which
raises a concern as these along with several other pesticides continue to be used world-wide and often in combination.

**CHAPTER 6: FUTURE DIRECTIONS**

For future investigations into the direct effects of pesticides on BAT function, analysis of the mechanism by which the other hits from this study effect BAT should be assessed. When further investigating the effects of CPF on BAT function, the effects of long term low dose exposure of CPF on BAT mitochondrial membrane potential should be determined. Also, the effects of CPF on BAT adipogenesis should also be investigated. Additionally, to determine how CPF may be decreasing complex IV activity and maximal respiration capacity, ROS production, antioxidant levels or changes in mitochondrial morphology should be examined. Furthermore, the effects of CPF metabolites (i.e., CPF oxon and TCP-y) on mitochondrial number, ETC enzyme activity, and membrane potential should be assessed. In order to determine how CPF is reducing UCP-1 expression levels other BAT specific markers that are not part of the β-adrenergic/cAMP signaling pathway should be assessed. Additionally, lipid accumulation should be assessed in exposed vs. control adipocytes in order to determine if changes to UCP-1 expression and mitochondrial function are causing increased lipid deposition.

Finally, although murine brown adipocytes have been used to assess BAT function and adipogenesis in other studies, using human adipocytes may more closely resemble a
human model. As CPF has been studied for its effects on other metabolic tissues of exposed animals (i.e., liver), the effects of low dose, long term exposure to CPF and other hits from this study on rodents, should also be examined in the context of BAT function.

CHAPTER 7: CONCLUSIONS

Due to the growing concern from scientists and health care professionals, alternative causes for metabolic diseases such as obesity or type-2 diabetes continue to be investigated. Over the decades, pesticide use has exponentially grown and current studies have highlighted the association between pesticide exposure and chronic diseases. However, there are limited studies on the association between pesticide exposure and metabolic diseases such as obesity. The results of this study add to the increasing evidence that pesticide exposure is associated with metabolic perturbations that may contribute to the obesity epidemic. The data from this study suggests a new mechanism by which atrazine, deltamethrin, and CPF can contribute to changes in metabolic function and imbalances in energy homeostasis. Furthermore, this analysis examined the mechanisms by which CPF may be inhibiting BAT function. While exposure to CPF has been shown to alter key components of metabolic function, no study has attributed these metabolic perturbations to disruptions in BAT thermogenesis. Moreover, this study adds to the numerous investigations on how organophosphorus insecticides can cause mitochondrial dysfunction and the importance in examining low-dose long-term exposure levels. Further studies should be made on the effects of pesticides and environmental
toxicants on BAT function as contributors to metabolic perturbations. As new compounds enter the market, their off-target effects are important to investigate as they can contribute to obesity, a global health concern and a leading cause of morbidity, mortality and health care expenditure.
CHAPTER 8: REFERENCES


32. Li, Y., Fromme, T., Schweizer, S., Schöttl, T., & Klingenspor, M. (2014). Taking control over intracellular fatty acid levels is essential for the analysis of
thermogenic function in cultured primary brown and brite/beige adipocytes. *EMBO reports*, e201438775.


CHAPTER 9: APPENDIX

Table 1. Primary screen: Pesticides and pesticide metabolites that reduce UCP-1 promoter stimulation

<table>
<thead>
<tr>
<th>Dose</th>
<th>1pM</th>
<th>1nM</th>
<th>1uM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2,4-D</td>
<td>-</td>
<td>↑</td>
<td>-</td>
</tr>
<tr>
<td>Atrazine</td>
<td>-</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Atrazine Mercapturate</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Desethyl Atrazine</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Diaminochlorotriazine</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Chlorpyrifos Oxon</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>TCP-y</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>3-Phenoxybenzoic acid</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>↓</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>AMPA</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Imidaclorpid</td>
<td>-</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>6-Chloronicotinic Acid</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Metam Sodium</td>
<td>↓</td>
<td>- (20%)</td>
<td>↓</td>
</tr>
<tr>
<td>MITC</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Permethrin</td>
<td>-</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>S-Metolachlor</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Table 2. Secondary screen: Pesticides that reduce BAT \textit{Ucp-1}

<table>
<thead>
<tr>
<th>Dose</th>
<th>1pM</th>
<th>1nM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Glyphosate</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>-</td>
<td>↓</td>
</tr>
<tr>
<td>Metam sodium</td>
<td>-</td>
<td>- 19%↓</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Pesticide Metabolites</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlorpyrifos Oxon</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Desethyl Atrazine</td>
<td>↓</td>
<td>-</td>
</tr>
</tbody>
</table>

Table 3. Tertiary screen: Pesticides that reduce UCP-1 protein expression

<table>
<thead>
<tr>
<th>Dose</th>
<th>1pM</th>
<th>1nM</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pesticides</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>↓</td>
<td>-</td>
</tr>
<tr>
<td>Chlorpyrifos</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Deltamethrin</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Imidacloprid</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th><strong>Pesticide Metabolite</strong></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Desethyl atrazine</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>